A MOLECULAR ANALYSIS OF THE INDUCTION
OF CLASS II MAJOR HISTOCOMPATABILITY ANTIGEN
EXPRESSION ON MURINE MACROPHAGES BY
INTERFERON-GAMMA AND ITS DOWN-REGULATION BY
INTERFERON-ALPHA/BETA AND DEXAMETHASONE

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ABSTRACT

Title of Dissertation: A Molecular Analysis of the Induction of Class II Major

Histocompatibility Antigen Expression on Murine Macrophages by

Interferon-γ and Its Down-Regulation by Interferon-α/β and

Dexamethasone

Diana Fertsch Ruggio, Doctor of Philosophy, 1989

Dissertation directed by: Stefanie N. Vogel, Ph.D., Associate Professor, Department of Microbiology

Previous studies have shown that the cell surface expression of class II major histocompatibility (MHC) antigens on murine macrophages (Ia antigens) is induced by interferon-gamma (IFN-γ) and down-regulated by interferon-alpha/beta (IFN-α/β) and dexamethasone (DEX). The studies described herein were performed in an attempt to examine the molecular mechanisms by which Ia antigens are induced on murine macrophages by recombinant IFN-γ (rIFN-γ) and how this induction process is down-regulated by the selected inhibitors, IFN-α/β and DEX. Steady-state analysis of total and cytoplasmic RNA revealed that rIFN-y induced a 5.7- to 6.5-fold increase in levels of Ia (e.g., A_{\alpha}-specific) mRNA. Increases in steady-state mRNA for A_{\beta} and E_{\alpha}. gene products were also observed in response to rIFN-y. Maximum accumulation of An-specific mRNA occurred 24 hr post-treatment and required the continued presence of rIFN- γ . Induction of A_{α} -specific mRNA was sensitive to the protein synthesis inhibitor, cycloheximide (CHX). Both the lag in time observed during the induction of maximal levels of Ia-specific mRNA by rIFN-y and the CHX sensitivity of this process support the hypothesis that IFN-γ may modulate Ia expression through an indirect mechanism, via the production of an intermediate protein(s). The steady-state studies also showed that both IFN- α/β and DEX reduced significantly, in a dose-dependent fashion, the levels of

 A_{α} -, A_{β} -, and E_{α} -specific mRNA induced by rIFN- γ and suggest that the inhibitors act pretranslationally. An increase in A_{α} -specific mRNA in response to rIFN- γ and a decrease in rIFN-γ-induced A_α-specific mRNA in response to IFN-α/β and DEX were observed in both C3H/HeJ (lipopolysaccharide-hyporesponsive) and C3H/OuJ (lipopolysaccharide-responsive) macrophages. A steady-state comparison of cytoplasmic and nuclear RNA species revealed that A_{α} -specific mRNA was induced by rIFN- γ in both cytoplasmic and nuclear compartments, and that this induction was antagonized by IFN- α/β and DEX in parallel. These findings suggested the possibility that regulation of I-region gene expression might be controlled at the level of transcription. To test the possibility that alterations in the rate of I-region gene transcription were responsible for the changes in steady-state levels of Ia-specific mRNA, nuclear "run-on" transcription experiments were performed. These experiments demonstrated that rIFN-y induced a 3.7-fold increase in the rate of I-region gene transcription. Furthermore, the addition of IFN-α/β or DEX to rIFN-γ-treated cultures led to a significant reduction in the rate of I-region gene transcription. The results of the nuclear "run-on" experiments demonstrated that rIFN-y increases the rate of I-region gene transcription and that the antagonists, IFN-α/β and DEX, decrease the rate at which these genes are transcribed. Taken collectively, the work presented herein demonstrates that the alterations in the steady-state levels of Ia-specific mRNA and Ia antigen are the result of changes in the rate of transcription of I-region genes.

A MOLECULAR ANALYSIS OF THE INDUCTION OF CLASS II MAJOR HISTOCOMPATIBILITY ANTIGEN EXPRESSION ON MURINE MACROPHAGES BY INTERFERON- γ AND ITS DOWN-REGULATION BY INTERFERON- α/β AND DEXAMETHASONE

by

Diana Fertsch Ruggio

Dissertation submitted to the Faculty of the Department of Microbiology

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To my husband, Michael, and to my Mom and Dad, Marlene and Charlie, for their unconditional love, support, and encouragement in all of the dreams that I pursue in my life.

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ABBREVIATIONS

APC, antigen-presenting cell

ATP, adenosine-5'-triphosphate

bp, basepair

BSA, bovine serum albumin

°C, degrees centigrade

Ca⁺², calcium

CaCl2, calcium chloride

cAMP, adenosine-3',5'-cyclic monophosphate

CD, cluster of differentiation

cDNA, complementary deoxyribonucleic acid

CHX, cycloheximide

Ci, curie

cm, centimeters

CO2, carbon dioxide

CsCl, cesium chloride

CTP, cytidine-5'-triphosphate

dATP, 2'-deoxyadenosine-5'-triphosphate

DAG, diacylglycerol

dCTP, 2'-deoxycytidine-5'-triphosphate

DEX, dexamethasone

dGTP, 2'-deoxyguanosine-5'-triphosphate

DHFR, dihydrofolate reductase

DNase, deoxyribonuclease

DNP-PLL, dinitrophenyl poly-L-lysine

DTT, dithiothreitol

dTTP, 2'-deoxythymidine-5'-triphosphate

EAE, experimental allergic encephalitis

EBSS, Earle's Balanced Salt Solution

EDTA, ethlenediaminetetraacetic acid

EGTA, ethylene glycol bis-(β-aminoethyl ether) N-N-N'-N'-tetraacetic acid

ELISA, enzyme-linked immunosorbant assay

EtBr, ethidium bromide

EtOH, ethanol

F(ab')2, bivalent antigen-binding fragment of an antibody

Fcy2aR, Fc receptor specific for IgG2a

Fcy2hR, Fc receptor specific for IgG2b

FCS, fetal calf serum

g, acceleration due to gravity; 9.8 m/sec²

GM-CSF, Granulocyte-Macrophage Colony Stimulating Factor

GRE, glucocorticoid response element

GTP, guanosine-5'-triphosphate

³H, titriated

H₂0, water

HEL, hen egg lysozyme

HEPES, N-2-hydroxy-ethyl piperazine-N'-2-ethanesulfonic acid

(H,G)-A--L, poly-(histidine,glutamic acid)-poly-D,L-alanine-poly-L-lysine

HLA, human leukocyte antigen

hr, hour

5-HT2, serotonin

Ia, I-region associated

IFN-interferon

IFN-α, interferon-alpha

IFN-α/β, interferon-alpha/beta

IFN-γ, interferon-gamma

IgG, immunoglobulin

IgG_{2a}, immunoglobulin G, subclass 2a

IgG_{2b}, immunoglobulin G, subclass 2b

Il 1, Interleukin 1

Il 2, Interleukin 2

Il 3, Interleukin 3

Il 4, Interleukin 4

IP₃, inositol-1,4,5-triphosphate

Ir, immune response

kDa, kilodaltons

kbp, kilobasepairs

KCl, potassium chloride

LFA, lymphocyte function associated

LPS, lipopolysaccharide

LT, lymphotoxin

LV, lentivirus

μCi, microcurie

μg, microgram

μl, microliter

μm, micron

m, meter

M, molar

max, maximum

MDP, muramyl dipeptide

2-ME, 2-mercaptoethanol

mg, milligram

MgCl₂, magnesium chloride

MHC, major histocompatibility complex

min, minutes

ml, milliliter

mm, millimeter

mM, millimolar

mmole, millimole

MnCl2, maganese chloride

MOPS, morpholinopropanesulfonic acid

mRNA, messenger ribonucleic acid

NaAc, sodium acetate

NaCl, sodium chloride

NaOH, sodium hydroxide

NaPP_i, sodium pyrophosphate

NE, norepinephrine

NF-Y, nuclear factor Y

N.I.H., National Institutes of Health

nm, nanometers

NP-40, Nonidet P-40

NT, not tested

1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃

OPD, o-phenylenediamine

OVA, ovalbumin

32P- phosphate-radiolabelled

PGEs, prostaglandins of the E series

PIP2, phosphatidylinositol-4,5-biphosphate

PLL, poly-L-lysine

PMA, phorbol myristate acetate

POMC, pro-opiomelanocortin

PPD, purified protein derivative

r, recombinant

RNA, ribonucleic acid

RNase, ribonuclease

S.D., standard deviation

SDS, sodium dodecyl sulfate

sec, seconds

S.E.M., standard error of the mean

SLE, systemic lupus erythematosis

SSC, sodium chloride-sodium citrate

TCA, trichloroacetic acid

TcR, T cell receptor

(T,G)-A--L, poly-(tyrosine,glutamic acid)-poly-D,L-alanine-poly-L-lysine

TNF-α, tumor necrosis factor alpha

Tris-HCl, tris (hydroxymethyl) aminomethane hydrochloride

tRNA, transfer ribonucleic acid

U, units

UV, ultraviolet

INTRODUCTION

As is true of many scientific investigations, the study of the regulation and control of immune responsiveness has spanned many decades. An abbreviated account of key breakthroughs in this field will be presented to provide the necessary background for the subject of this dissertation. My work has focused on the study of molecular mechanisms which modulate the expression of class II major histocompatibility (MHC) antigens on macrophages.

GENERAL FEATURES OF IMMUNE RESPONSIVENESS

In the late 19th and early 20th Centuries, clinical observations led to the notion that an individual's susceptibility or resistance to diphtheria may be inherited (Jacobi, 1877; Hirszfeld et al., 1924). Evidence from several laboratories in the 20th Century, in fact, confirmed the hypothesis that inheritance played a role in the immune responsiveness to diphtheria with the demonstration that the ability to produce antibody to the Corynebacterium diphtheriae toxin was inherited dominantly in a simple, Mendelian fashion (Rosling, 1929; Schiebel et al., 1943). At approximately the same time, researchers were also studying the role of inheritance in the susceptibility and resistance to other human, livestock, and experimentally-induced diseases. These included tuberculosis, leprosy, brucellosis, and typhoid syndrome of the mouse. The role of inherited immune responsiveness, in terms of a humoral component, was not demonstrated in these early disease models and suggested that host factors, other than the ability to produce antibody, might be operational in an animal's innate susceptibility or resistance to these diseases (reviewed in Gowen, 1948).

These early findings provided the impetus for investigators to examine the potential genetic basis for immune responsiveness to other antigens. The mouse and guinea pig were popular experimental systems to study because of the availability of inbred strains. Structurally simple proteins and synthetic peptides, both hapten-conjugated linear homopolymers and unconjugated branched heteropolymers, were widely-employed antigens in these systems because the immunized inbred animals displayed distinct patterns of responsiveness. There were generally two categories of response patterns observed, i.e., "responders" and "non-responders". These categories reflected the animal's ability to develop humoral immunity (i.e., the capacity to synthesize significant levels of specific antibody) and/or cellular immunity (i.e., the capacity to generate an antigen-specific, delayed-type hypersensitivity response). The ability of random-bred Hartley guinea pigs to respond to the antigen, poly-L-lysine covalently coupled to dinitrophenyl (DNP-PLL), was one of the first structurally-defined antigenic systems studied (Kantor et al., 1963; Levine and Benacerraf, 1965). These studies analyzed the immunogenicity of this synthetic peptide, in terms of its ability to promote specific antibody synthesis and to activate specific T cell populations, and confirmed the early observation that responsiveness was a dominantly-inherited trait. Green et al. (1966) extended this work and demonstrated that non-responsiveness was not due to the failure of B cells to synthesize anti-DNP antibodies, as evidenced by the ability of animals to respond to DNP-PLL complexed with bovine serum albumin (BSA), but was more likely due to the failure of T cells to recognize the PLL carrier determinant. Benacerraf and McDevitt (1972) speculated that the genes which control the immune response patterns to specific antigens ("immune response" or Ir genes) might be expressed in T cells and proposed that the products of these genes might be antigen-specific T cell receptors (TcR). Therefore, failure to express a particular TcR gene might result in an animal's inability to provide appropriate "help" from T cells, in the process of the activation and production of antibody by B cells.

McDevitt and Sela (1965; 1967) demonstrated that immune responsiveness was

regulated in an antigen-specific manner. They showed that modifications in branched synthetic polypeptides (i.e., a substitution of histidine for tyrosine) could radically alter the ability of certain inbred mouse strains to respond to a particular antigen. For example, the CBA mouse strain is a low responder to the synthetic antigen poly-(tyrosine, glutamic acid)-poly-D,L-alanine--poly-L-lysine [(T,G)-A--L] and strains on a C57BL background are high responders. However, immunization of CBA and C57BL strains with a similar antigen that has histidine substituted for tyrosine [(H,G)-A--L] completely reversed the pattern of responsiveness. These experiments also demonstrated that the specificity of control resides in the antigenic determinant [i.e., the (T,G) or (H,G) portion of the antigen] and not in the carrier determinant.

FUNCTIONAL ASPECTS AND IDENTIFICATION OF THE GENE PRODUCTS RESPONSIBLE FOR IMMUNE RESPONSIVENESS

Experiments which associated Ir genes with the major histocompatibility locus (MHC or H-2 in the mouse) provided revolutionary insights into the function of these genes (McDevitt and Chinitz, 1969; McDevitt and Tyan, 1968; Ellman et al., 1970). The H-2 locus was originally identified by Gorer et al. (1948) and Snell and Bunker (1946) who demonstrated the involvement of the MHC locus in the transplantability of tumor allografts between different mouse strains. They subsequently demonstrated that the MHC locus consisted of a cluster of many genes and, hence, the designation "MHC complex" was adopted. Initial experiments (McDevitt and Chinitz, 1969) demonstrated that strains of a common "MHC-type" responded similarly to a particular antigen. For example, when certain strains (H-2^k) were immunized with the branched polypeptide (T,G)-A-L, they produced a high antibody response, whereas other strains (H-2^d and H-2^b) responded poorly. Adoptive transfer studies (McDevitt and Tyan, 1968), in which responder spleen

cells (from a high responder x non-responder F₁ animal) were transferred into irradiated non-responder recipients, together with H-2 typing studies, further supported the physical association between Ir genes and the MHC locus. These studies showed that the post-transfer recipients acquired the capacity to generate an antibody response comparable to that of F₁ or responder animals. Using recombinant inbred strains of mice, McDevitt et al. (1972) confirmed previous observations which associated Ir phenotype and MHC type and demonstrated that the ability of mouse strains to respond to a series of synthetic polypeptide antigens mapped to a region between the H-2K locus and the Ss-Slp locus (serum substance locus within the MHC complex on chromosome 17). Shortly thereafter, Shevach and Rosenthal (1973) suggested that Ir genes might be expressed in macrophages, given their observations that the presentation of antigen to T cells was restricted such that only macrophages from a responder animal could "present" antigen to T cells (derived from an F₁ animal). From these studies, the term "MHC restriction" was coined and implied that for a T cell to be activated, it must recognize foreign antigen in association with "self" MHC-derived antigens (discussed below) expressed on antigen-presenting cells (APCs), such as macrophages. Subsequently, the activated T cells provide "help" and stimulate antigen-specific antibody-producing B cells. In this system, Shevach and Rosenthal postulated that "non-responsiveness" to particular antigens was the result of a failure to present antigen appropriately, rather than a defect in the T cell repetoire. Prior to these studies, there had not been any suggestion that the "antigen nonspecific" macrophage might be involved in immune responsiveness.

Throughout the 1970's this work was extended in many different directions. Katz et al. (1973) demonstrated that B cells from responder animals only were capable of presenting antigen to (responder x non-responder) F₁ T cells. In chimeric animals, which consist of a mixture of cellular genotypes, Kappler and Marrack (1978) showed that non-responder T cells that were raised in an environment which contained both high and low responder APCs were capable of providing the same degree of "help" to

responder APCs as T cells raised in an environment which contained only responder APCs. In addition, these experiments demonstrated that immune responsiveness was not controlled by the genotype of the T cell. Therefore, it was unlikely that the Ir genes encoded an antigen-specific TcR, as originally proposed by Benacerraf and McDevitt (1972). These findings are also consistent with the hypothesis that Ir gene products are expressed on APCs. Other investigators were interested in the mechanisms(s) by which the Ir phenotype was influenced developmentally. Chimeric studies (Singer et al., 1981) and thymic graft studies (Miller et al., 1979; Hedrick and Watson, 1979) revealed that the Ir phenotype was determined by the host environment, and in particular, by the thymus.

To identify the products of Ir genes, antibodies against this genetic region (called the "I-region") were generated by immunizing recombinant inbred strains with spleen cells from different recombinant inbred strains which exhibited different patterns of responsiveness to synthetic antigens (Cullen and Schwartz, 1976; Cresswell, 1977; Clement et al., 1978). The antisera reacted predominantly with macrophages and B cells of the immunizing strain (Shreffler and David, 1975; reviewed in Schwartz, 1976), and provided further support for the proposal of Shevach and Rosenthal that the products of the Ir genes are expressed on APCs. The products recognized by these serological reagents were subsequently named "I-region associated" (Ia) molecules or "class II MHC" antigens. Functional studies in which these antibodies were employed were undertaken to determine the role of these products in the immune response. For example, Shevach et al. (1972) demonstrated that only antibodies directed against Ia molecules of a responder strain blocked the response to antigen. Definitive proof that Ia molecules were, in fact, the products of the Ir genes came primarily from studies of the spontaneously occurring I-region mutant, bm12, of the H-2b haplotype of the C57BL/6 mouse strain (McKenzie et al., 1979; Hansen et al., 1980). The bm12 mutant is immunologically defective as evidenced by: (i) its inability to respond to antigens to which the wild-type C57BL/6 strain responds; (ii) its inability to provide help in the generation of cytotoxic T cells; (iii) its

STRUCTURAL FEATURES OF CLASS II MHC ANTIGENS AND THE MOLECULAR ORGANIZATION OF CLASS II MHC GENES

With the discovery that the Ia antigens were the cell surface molecules responsible for MHC restriction in the process of antibody production, many scientists became interested in elucidating the structural organization of the Ia molecule, the molecular organization of the genes which encode Ia molecules, and the processes which underlie macrophage-T cell interactions. Using antisera generated against Ia molecules from different strains, several investigators determined the structure of these molecules by immunoprecipitation and electrophoretic separation methods (reviewed by Strominger et al., 1981; Clement and Shevach, 1981). Ia molecules are cell surface glycoproteins composed of an α -chain (33 - 35 kDa) and a β -chain (27 - 29 kDa) which associate noncovalently. A third glycoprotein, the nonpolymorphic invariant chain (I_i), has been shown to associate noncovalently with the α and β chains in intracellular membranes (Sung

and Jones, 1981; Kvist et al., 1982). I; neither associates with Ia antigens isolated from plasma membranes nor is it present as a soluble cytoplasmic protein (Sung and Jones, 1981). As shown in Figure 1A, each polypeptide chain of an Ia molecule contains 2 extracellular domains. The domains are referred to as α_1 and α_2 in the α chain and β_1 and β_2 in the β chain. In addition, each polypeptide chain contains a hydrophobic transmembrane domain and a hydrophilic intracytoplasmic domain. There are two principal types of Ia molecules expressed in the mouse: I-A and I-E. The I-A molecule consists of and A_α and an A_β chain and the I-E molecule consists of an E_α and an E_β chain. There are strong homologies between subregion $A_{\beta 1}$ and subregion $E_{\beta 1}$ (Larhammar et al., 1982) and similarly, between $A_{\alpha 1}$ and $E_{\alpha 1}$ (Benoist $\underline{et}~\underline{al}.,$ 1983a). The α_2 and β_2 domains show strong homology with β_2 -microglobulins, α_3 domains of class I MHC antigens, and constant regions of immunoglobulins (Kaufman et al., 1984). The α_1 and β_1 domains show extensive allelic variability among several haplotypes examined (Benoist et al., 1983b; Robinson et al., 1983). It had also been demonstrated that these domains contain the residues involved in cell-cell recognition (Folsom et al., 1985; Rask et al., 1985; Germain and Malissen, 1986; Ronchese et al., 1987).

In the human system, three major types of class II MHC antigens have been described. They are designated human leukocyte antigen (HLA) -DR, -DP, and -DQ. Structurally, these antigens are very similar to Ia antigens. The HLA-DQ gene products share significant homology with the I-A molecules, whereas the HLA-DR gene products are more homologous with I-E molecules (Hyldig-Nielsen et al., 1983; Saito et al., 1983). In contrast to the murine system, where all of the α and β chains chains exhibit a relatively high degree of allelic variability, DR $_{\beta}$, DP $_{\beta}$, DQ $_{\beta}$, and DQ $_{\alpha}$ are the only polymorphic alleles in the human system (Schenning et al., 1984).

The genetic organization of the I-region has been examined extensively at a molecular level (reviewed in Steinmetz and Hood, 1983). In both murine and human systems, each domain region within the protein is encoded by a distinct exon (Figure 1B).

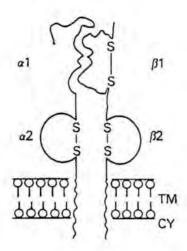
Figure 1. Representation of the organization of I-region loci and class II MHC genes and antigens.

A. Organization of a prototype class II MHC molecule. The abbreviations are as follows: $\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$, the extracellular domains of the α and β proteins, respectively; TM, transmembrane segment; CY, cytoplasmic tail; and, S-S, intradomain disulfide bond. Reproduced with permission from the authors (Germain and Malissen, 1986).

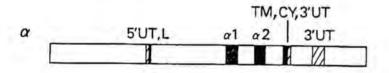
B. Organization of class II MHC α - and β -chain genes. The abbreviations are as follows: 5'UT, 5'-untranslated region exon; L, leader sequence exon; α 1, α 2, β 1, β 2, the extracellular domain exons of the α - and β -chain genes, respectively; TM, transmembrane domain exon; CY, cytoplasmic domain exon; and, 3'UT, 3'-untranslated region exon. Introns are designated as open blocks, exons as shaded blocks, and untranslated regions as hatched blocks. Reproduced with permission from the authors (Germain and Malissen, 1986).

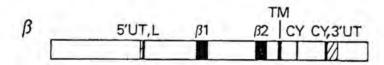
C. Organization of the I-region genes. The arrows indicate the direction of transcription (5' to 3') of the indicated loci. Reproduced with permission from the authors (Devlin et al., 1984).

A. Class II MHC Antigen Organization

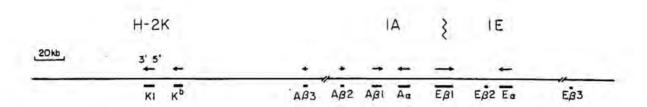


B. Class II MHC Gene Organization





C. I-Region Gene Organization



This domain-exon correlation is very reminiscent of the immunoglobulins and the genes which encode these structures. It has been proposed that class II MHC molecules belong to a family of evolutionarily-related genes (which is referred to as the "immunoglobulin gene superfamily" and includes Thy-1, Lyt-2, class I MHC antigens, β_2 -microglobulin, immunoglobulins, and TcRs) based on this domain-exon organization and significant nucleic acid sequence homologies between class II MHC antigens and these other genes (Kaufman et al., 1984). A comparison of the molecular organization of the α and β chain loci reveals the following commonalities: (i) an exon which encodes a leader peptide for the appropriate targeting of the peptide to the cell membrane; (ii) an exon which encodes the α_1 or β_1 domain; and, (iii) an exon which encodes the α_2 or β_2 domain. There are differences between α and β genes at their 3' ends which include the following: (i) α genes have a single exon which encodes for a transmembrane domain, an intracytoplasmic domain, and part of an untranslated region, in addition to a separate exon which encodes the remainder of the untranslated region, and (ii) β genes have a transmembrane domain which is encoded by a distinct exon, an intracytoplasmic domain encoded by another exon, and a region of an intracytoplasmic domain and an untranslated domain which are encoded by a single exon (Figure 1B; Germain and Malissen, 1986). In the mouse I-region, two genetic subregions, I-A and I-E, have been identified (Figure 1C). The I-A genetic subregion contains the loci which encode the Aa, AB, and EB chains, whereas the I-E genetic subregion contains the locus which encodes the Ea chain. Three additional loci, E_{β2}, A_{β2}, and A_{β3} have been identified (Larhammar et al., 1983; Steinmetz et al., 1982) and the function of the $E_{\beta2}$ and $A_{\beta3}$ genes is not yet clear. Wake and Flavell (1985) have demonstrated that the AB2 gene is transcribed and translated in splenocytes and in a B cell hybridoma, but not in macrophages. In the human system, the total number of class II MHC genes has not yet been determined; however, three main genetic subregions have been described (HLA-DR, -DP, and -DQ). It appears that each of these genetic subregions contain α and β chain loci clusters similar to that in the mouse (Trowsdale et al., 1984;

Wiman et al., 1982; Korman et al., 1982).

GENERAL ASPECTS OF THE NATURE OF THE T CELL-MACROPHAGE INTERACTION

In addition to the chemical and molecular analysis of class II MHC antigens and the loci which encode them, investigation into the mechanisms which underlie the interaction between foreign antigen, the macrophage, and the T cell have been pursued avidly. The nature of the antigen-processing event has been studied extensively (reviewed in Unanue and Allen, 1987). Using the complex antigen, Listeria monocytogenes, Zeigler and Unanue (1981) characterized the macrophage antigen-processing event as one which consists of a rapid antigen internalization phase, followed by a delayed phase of T cell-macrophage binding. The period from uptake of antigen to the binding of the T cell to the macrophage was referred to as the "processing phase". The processing phase was shown to be sensitive to chloroquine and ammonium chloride (i.e., lysozomotropic agents that raise the pH within acidic vesicular compartments) which implies that the events during this phase involve the degradation of antigen in an acidic endosomal compartment. Other, less complex antigens, including ovalbumin (OVA) and hen egg lysozyme (HEL), were similarly shown to require degradation or fragmentation in order to be presented to T cells (Chesnut et al., 1982; Allen and Unanue, 1984). Shimonkevitz et al. (1983) demonstrated that peptide fragments of OVA could be presented by paraformaldehyde-fixed macrophages (a treatment which renders them incapable of antigen uptake and processing) and their data suggest that these peptides could interact appropriately with Ia molecules on the cell surface. For HEL, the antigenic determinant that is recognized by the T cell is inaccessible on the native molecule. Therefore, the antigen-processing event is critical to the initiation of an immune response in that it actually produces the antigenic determinants that will be

presented to the immune system. The nature of the association between the processed antigen and the Ia molecule was also studied by Unanue and his colleagues. Babbitt et al. (1985) demonstrated that Ia molecules from responder haplotypes bind specifically to a particular fragment of HEL. The binding is saturable and specific in that it is not observed with Ia molecules from non-responder haplotypes. Unanue and Allen (1987) proposed that processed antigen and an Ia molecule may interact in a vesicular compartment which is subsequently shuttled to and inserted into the cell membrane. In this model, both newly synthesized Ia molecules, as well as recycled membrane Ia components, could interact with processed antigen. Although the invariant chain has been shown to be unnecessary for the membrane expression of Ia molecules (Miller and Germain, 1986; Sekaly et al., 1986), this chain may play a role in the association and rate of transport of α chains, β chains, and possibly processed antigen in these acidic intracellular compartments (Claesson-Welch and Peterson, 1985).

In addition to the antigen-processing event, the role of the soluble factor

Interleukin 1 (IL 1) in the activation process has also been studied extensively. IL 1 is released from macrophages following contact with helper T cells and one of its targets is the helper T cell (reviewed in Unanue and Allen, 1987). IL 1 causes an increase in receptors for the T cell growth factor, Interleukin 2 (IL 2), and also stimulates the production of IL 2. It is believed that a membrane-associated form of IL 1 plays a critical role in T cell activation, as evidenced by the ability of metabolically inert, formaldehyde-fixed macrophages to present antigen and activate T cells (Kurt-Jones et al., 1985).

Interferon-gamma (IFN-γ), a product of activated T cells, has also been shown to enhance the ability of LPS to induce IL 1 secretion (Arenzana-Seisdedos and Virelizier, 1983). The relationship between the macrophage and the helper T cell is characterized by reciprocity and amplification: after interacting with the helper T cell, the macrophage provides a stimulatory signal (IL 1) to the helper T cell, and then the activated helper T cell can provide a stimulatory signal (e.g., IFN-γ) to the macrophage to generate a very

favorable environment for the activation and subsequent induction of T cell effector activity.

Other laboratories have researched extensively the nature of the receptor on the T cell that recognizes foreign antigen in association with class II MHC antigen (for review see Schwartz, 1985). From experiments described earlier (Shevach and Rosenthal, 1973: Katz et al., 1973; Kindred and Shreffler, 1972), it was concluded that the TcR has dual specificity; that is, this receptor is restricted in that it recognizes foreign antigen only in the context of self-MHC antigen on APCs. The majority of TcRs on peripheral T cells are glycoprotein heterodimers which consist of an α and a β chain joined convalently by a disulfide bond (reviewed in Allison and Lanier, 1987). It is currently believed that the polypeptide chains of the TcR (α and β) associate to form a single binding site that can interact with both foreign antigen and class II MHC antigen (Kronenberg et al., 1986; Allen et al., 1987; Marx, 1987). The TcR is associated with a collection of nonpolymorphic membrane proteins, the "CD3 complex", and it is believed that this complex may be responsible for the transmission of the signal for the TcR-processed antigen/class II MHC antigen interaction across the plasma membrane. In addition, other molecules, including cluster of differentiation antigen 4 (CD4 or L3T4) and lymphocyte function associated antigen 1 (LFA-1), have been implicated as playing a role in the initial, antigen non-specific binding interactions between T cells and APCs (Allison and Lanier, 1987; Wilde et al., 1983; Marrack et al., 1983; Dembic et al., 1986). Experiments are ongoing to examine the precise nature of the interaction between the foreign antigen/class II MHC antigen complex and the TcR. Ronchese et al. (1987) have demonstrated that there are two unique sites on the Ia molecule; one that is involved in binding to the TcR and another that is involved in antigen binding. Allen et al. (1987) have distinguished the amino acid residues of a determinant of the antigen HEL as either Ia contact residues or TcR contact residues by using a panel of peptides with single residue substitutions in T cell activation and antigen-presenting competition assays. From the study, they have generated a

three-dimensional computer image of the HEL determinant which predicts that the contact sites for the Ia molecule and the TcR are oriented on opposite faces of an α-helix. These findings are consistent with the structure of a human class I MHC antigen demonstrated using X-ray crystallographic methods (Bjorkman et al., 1987a; 1987b)

The elucidation of the events that occur during the interaction of antigen with the macrophage and the macrophage with the helper T cell have provided the basis for which to examine the nature of the defect in immune responsiveness. The two major models that have emerged to explain immune response defects are: (i) the presentation model, and (ii) the receptor recognition model (Schwartz, 1985). The presentation model proposes that a defect in the capacity of APCs to present antigen appropriately in the context of self-class II MHC antigen results in the failure to activate antigen-specific T cell clones. Theoretically, a defect in presentation could occur at the level of antigen processing or binding. It was originally postulated that responder animals were capable of degrading antigen effectively, whereas non-responders were not (Levine et al., 1963). Data in support of this theory were lacking and Levine and Benacerraf (1964) provided direct evidence to suggest that there was no difference in the ability of non-responder and responder guinea pigs to internalize and process several synthetic polypeptide conjugates. There is evidence in support of antigen binding as a level at which an immune response defect may occur. Evidence presented earlier (Babbitt et al., 1985), demonstrates that Ia molecules from non-responder haplotypes are incapable of binding the antigen (to which the animal is hyporesponsive), whereas Ia molecules from responder haplotypes are fully capable of binding the antigen. It has been postulated by Babbitt et al. (1985) that the affinity of binding between foreign antigen and self-class II MHC antigen may also contribute to an effective interaction. Furthermore, their observations support the hypothesis that binding between antigen and a class II MHC molecule is necessary for antigen presentation and subsequent T cell activation. Failure of Ia alleles to interact effectively with processed antigen may result in immune hyporesponsiveness. The

receptor recognition model of immune response gene defects operates at the level of the TcR. It has been proposed that a failure to respond to certain antigens may result from the absence of antigen-specific T cell clones (Schwartz, 1985). This could result from: (i) a failure of the germline DNA to encode that particular T cell receptor and/or (ii) active deletion of those T cell clones during development due to a cross-reactivity with a self-antigenic determinant (Jerne, 1971). Either of these scenarios could result in a "hole" in the T cell repertoire.

These models and the research to date on immune regulation have created more questions than answers. Of particular relevance to the topic of this dissertation are questions which focus on the mechanisms of regulation of class II MHC antigen expression on the surface of APCs. For example, a defect in antigen presentation, at the level of antigen-class II MHC molecule binding, has been shown to result in immune hyporesponsiveness (Babbitt et al., 1985). However, if there were a defect in the expression of class II MHC antigens, one might also predict that alterations in immune responsiveness might occur. An underexpression or an overexpression of class II MHC antigens on APCs could result in conditions of immune hyporeactivity and hyperreactivity, respectively. The latter possibility has been a major focus of recent investigations in the field of autoimmunity. As a result, the regulation of class II MHC antigen expression in normal and inflammatory environments has been studied extensively.

NATURE OF THE BASAL AND INDUCED LEVELS OF CLASS II MHC ANTIGEN

Since the appropriate presentation of antigen to T cells is dependent upon the expression of class II MHC antigen, one can imagine the potentially disasterous events that might result from the aberrant expression of class II MHC antigens. Thus, it is not surprising to find that the expression of these antigens on macrophages and other cell types

is highly regulated. Beller and Unanue (1981) observed that Ia antigen expression on

The induction of Ia antigens on cells which normally express low levels of Ia antigen is also highly regulated. The efforts of many laboratories have demonstrated that a principal inducer of Ia antigens on macrophages is IFN-γ, a secreted product of activated T cells. Early studies by Steinman et al. (1980) demonstrated that a soluble factor present in supernatants from Trypansoma cruzi-treated spleen cell cultures (prepared from

Trypanosoma cruzi-infected animals) was (were) capable of converting a macrophage population that was initially < 5% Ia positive to one with > 95% Ia-positive cells. These findings were subsequently confirmed and extended by Steeg et al. (1980), who identified a component(s) in Concanavalin A (Con A)-stimulated spleen cell supernatants which was (were) capable of inducing expression of Ia antigen on Ia-negative peritoneal exudate macrophages in vitro. These in vitro findings were supported in vivo by studies which demonstrated the ability of culture fluids generated from Listeria-immune peritoneal exudate cells incubated with heat-killed Listeria to induce a population of peritoneal exudate macrophages that were > 50% Ia positive when administered intraperitoneally (Scher et al., 1980). The Ia-inducing activity present in the Con A-activated spleen cell supernatants was purified by a variety of chromatographic procedures and was shown to be antigenically and biochemically related to IFN-y (Steeg et al., 1982a). Conversely, IFN-y, which had been highly purified from a variety of T cell sources, was shown to mediate the induction of Ia antigen on APCs in vivo (Nakamura et al., 1984; Skoskiewicz et al., 1985). In contrast, IFN-alpha (IFN-α) and IFN-beta (IFN-β) were found not to induce class II MHC antigens on human or murine macrophages (Basham and Merigan, 1983; Rosa et al., 1983; Vogel et al., 1983; Kelley et al., 1984). Subsequent cloning of the murine IFN-y gene (Gray and Goeddel, 1983) and purification of its recombinant product (rIFN-y) allowed for the unambiguous demonstration that IFN-y induces Ia antigen on murine macrophages and macrophage cell lines (King and Jones, 1983; Wong et al., 1984a).

Since the elucidation of IFN-γ as a chief inducer of macrophage Ia antigen expression in antigen- or mitogen-stimulated spleen cell supernatants, other substances have also been identified which induce class II MHC antigen expression or which synergize with IFN-γ to induce class II MHC antigen expression. Groenwegen et al. (1986) demonstrated the presence of a factor with class II MHC antigen-inducing capacity, distinct from IFN-γ, that is produced in unstimulated human leukocyte cultures, mixed leukocyte cultures, and lectin-stimulated leukocyte cultures. Recent studies have shown

that Interleukin 4 (IL 4) also induces Ia antigen expression on murine peritoneal macrophages (Crawford et al., 1987), as well as on bone marrow-derived macrophages (Zlotnick et al., 1987). The recombinant monokine, human tumor necrosis factor-ox (rTNF-α), was shown to induce cell surface expression of Ia antigen minimally, as well as A_α-specific mRNA, in the murine myelomonocytic cell line, WEHI-3 (Chang and Lee, 1986). However, in combination, rIFN-γ and rTNF-α induced Ia antigen expression in a synergistic manner, both at the level of protein and steady-state RNA (Chang and Lee, 1986). This synergy between TNF and rIFN-γ has also been demonstrated in human islet cells (Pujol-Borrell et al., 1987) and murine islet cells (Wright et al., 1988). In this system, IFN-γ alone failed to induce class II MHC antigen expression; however, induction of class II MHC antigen expression was observed when cells were treated with IFN-y in combination with either TNF or the closely related lymphokine, lymphotoxin (LT). Recently, Weetman and Rees (1988) have shown that TNF-α, although incapable of inducing Ia antigen expression alone, can enhance the ability of IFN-γ to induce Ia antigen expression on thyroid cells. The hormone, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], has also been shown to synergize with IFN-y to increase Ia expression on WEHI-3 cells (Morel et al., 1986). The bacterial cell product, muramyl dipeptide (MDP), alone or in combination with IFN-y, has also been shown to induce class II MHC-specific mRNA (HLA-DR mRNA) in human monocytes (Vermeulen et al., 1987). Recent evidence has been presented which demonstrates that Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) enhances the antigen-presenting ability of splenic macrophages and bone marrow-derived macrophages. In these studies, Morrissey et al. (1987) also demonstrated that treatment of splenic macrophages, which consist of an already high proportion of Ia-positive cells (Cowing et al., 1978), with GM-CSF, leads to increased density of Ia antigens per cell, but does not lead to an absolute increase in numbers of Ia-positive cells. Falk et al. (1988) have shown that bone marrow progenitors cultured in the presence of GM-CSF differentiate into macrophages which express high basal levels of Ia antigen (by

measurement of Ia-specific protein and RNA) and that these already high levels are unaltered by additional treatment with IFN-γ. Lastly, certain viruses, including coronavirus (Massa et al., 1986), Visna-Maedi Virus (Kennedy et al., 1985), Herpes Simplex Virus (Howie et al., 1986), and Harvey and Kirsten Murine Sarcoma Viruses (Albino et al., 1986) have been shown to increase Ia antigen expression on macrophage and non-macrophage populations.

POTENTIAL ROLE OF ABERRANT CLASS II MHC ANTIGEN EXPRESSION IN DISEASE

The ability of IFN-y to induce Ia antigen expression is not limited to macrophages and antigen-presenting cells. IFN-y has also been shown to induce class II MHC antigens on a number of different cell types including human vascular endothelial cells (Pober et al., 1983; de Waal et al., 1983; Collins et al., 1984; Groenewegen et al., 1985), dermal fibroblasts (Collins et al., 1984), myeloid cells (Wong et al., 1983), thymic epithelial cells (Lo and Sprent, 1986; Papiernik et al., 1986), melanoma cell lines (Houghton et al., 1984), carcinoma cell lines (Schwartz et al., 1985), B lymphoid cell lines (Wong et al., 1983), mast cell lines (Wong et al., 1984a), and astrocytes (Wong et al., 1984a). In certain instances, the induction of class II MHC antigen expression by IFN-y on cells other than those typically involved in the generation of a normal immune response has led to some interesting observations and models about the role of these induced populations in autoimmune states (Pujol-Borrell et al., 1987; Bottazzo et al., 1986) and graft rejection processes (Lampert et al., 1981; de Waal et al., 1983). Bottazzo et al. (1983) suggested that the induction of autoimmunity may reflect a multi-stage process which involves the aberrant expression of class II MHC antigens on non-immune cells, the presentation of self antigens, and the subsequent activation of autoreactive T cells. For example, the induction

of class II MHC antigens on thyroid epithelial cells may enhance the presentation of thyroid self-antigens that subsequently activate thyroid antigen-specific T cell clones and thus contribute to an acquired state of "self-reactivity" seen in autoimmune thyroiditis (Bottazzo et al., 1983).

Systemic lupus erythematosis (SLE), a disease characterized by the presence of antibodies to nuclear antigens and an immune complex-mediated glomerulonephritis, has a significantly higher prevalance in humans with HLA-DRw2 or HLA-DRw3 antigens (Gibofsky et al., 1978), and in mice of the H-22 haplotype (Knight et al., 1978; Yoshida et al., 1981). Adelman et al. (1983) have shown that administration of anti-Ia (I-A) antibodies to (NZB x NZW)F₁ animals suppressed the normal induction and progression of renal disease in this murine model of SLE. In addition, in vivo administration of antibodies to Ia antigens in two additional diseases with autoimmune complications, namely experimental allergic encephalitis (EAE; Steinman et al., 1981; Sriram and Steinman, 1983) and experimental autoimmune myasthenia gravis (Waldor et al., 1983), were found to suppress markedly the clinical manifestations of these two diseases. These experiments provide evidence for the involvement of class II MHC antigen expression in the susceptibility of these diseases and for the possibility of using antibodies against immune response gene products in the treatment of these autoimmune diseases. In addition, Jacob et al. (1987) have directly demonstrated a role for IFN-γ in the generation of murine lupus nephritis and have shown that treatment of (NZB x NZW)F₁ animals in vivo with purified anti-IFN-y monoclonal antibody can delay both the onset of severe proteinuria and the appearance of anti-nuclear antibodies. Although the mechanism of action of IFN-γ in the generation of this autoimmune disease is unclear, one could propose that IFN-γ induces class II MHC antigen expression on cells that do not normally express these antigens, which in turn, leads to the presentation of self antigens and the subsequent activation of autoreactive T cells. In another autoimmune disease model that involves renal injury, Kelley and Roths (1982) have demonstrated that macrophage Ia antigen expression is

increased in MRL-lpr mice with concomitant lymphoproliferation. One could postulate that in this model, lymphoproliferation provides the constant source of lymphokines for the induction of Ia antigen expression which triggers the autoreactive cascade. Lu and Unanue (1982) have suggested that spontaneous production of a T cell lymphokine(s) may be responsible for enhanced Ia antigen expression in MRL-lpr mice.

Studies of the autoimmune disease, Grave's thyrotoxicosis (Bottazzo et al., 1983), have provided additional evidence in support of a "self-reactive" hypothesis of autoimmunity. Grave's thyrotoxicosis (a hyperthyroid condition) has been characterized, in part, by the production of thyroid-stimulating antibody that binds to the receptor for thyroid stimulating hormone. Thyrocytes isolated from patients with this disease have been shown to express class II MHC antigens constitutively. Londei et al. (1984) have demonstrated that these class II MHC antigen-expressing thyrocytes are capable of presenting a peptide fragment of the Influenza A hemagglutinin molecule to antigen-specific T cell clones. Although the thyrocytes were only capable of presenting "processed" antigens, and not unprocessed, native antigens, it is possible that they are still able to present self-antigens that are already localized in the cell membrane.

Findings from work on type 1 diabetes mellitus (Pujol-Borrell et al., 1987; Foulis and Farquharson, 1986), in which pancreatic beta (β) cells have been shown to be class II MHC antigen-positive, are also supportive of the involvement of aberrantly expressed class II antigens by endocrine cells in the immunopathology of autoimmune disease. These investigators have demonstrated in an in vitro system that class II MHC antigens are induced on pancreatic β -cells by a combination of IFN- γ and TNF or LT. This "two-signal" requirement for the induction of class II MHC antigens may serve as a "safeguard" to limit aberrant class II MHC antigen expression. The mechanism of β -cell destruction has not yet been determined, but one could postulate that the expression of Ia antigen on pancreatic β -cells initiates an immune response directed against β -cell antigens via the production of anti- β -cell antibodies and/or mediated through the generation

of β -cell-specific cytotoxic T cells that eventually leads to their destruction. Recently, Campbell <u>et al.</u> (1988) have shown that IFN- γ potentiates the severity of diabetes (i.e., increased hyperglycemia and weight loss) in mice treated with the β -cell toxin, streptozotocin. Their data have suggested that IFN- γ enhances the expression of both class I and II MHC antigens on β -cells of streptozotocin-treated animals, thus making these cells a likely target in the autoimmune process. In addition, Shizuri <u>et al.</u> (1988) have demonstrated that the administration of antibodies against the L3T4 antigen found on helper T cells to non-obese diabetic mice, which spontaneously develop diabetes, prevents hyperglycemia and delays the lymphocytic infiltration into islets that typically follows β -cell destruction. The efficacy of anti-L3T4 immunotherapies have also been shown in other autoimmune diseases including SLE (Wofsy and Seaman, 1987), EAE (Waldor <u>et al.</u>, 1985), and type II collagen-induced arthritis (Ranges <u>et al.</u>, 1985). The mode of action of this serologic reagent is unclear.

Aberrant class II MHC antigen expression has also been implicated in the graft rejection process. Observations from de Waal et al. (1983) have suggested that Ia antigens induced on vascular endothelial skin allografts (presumbably by activated T cell products) render these cells the eventual targets of the rejection process. Similar findings have been observed in a graft-versus-host disease model. Specifically, Lampert et al. (1981) have shown that keratinocytes isolated from rats with graft versus host disease (e.g., induced experimentally by injecting histoincompatible lymphocytes into irradiated animals) have significantly increased levels of cell surface Ia antigen. Coincidently, they have demonstrated in this experimental model of graft-versus-host disease, that keratinocytes are the main targets of the rejection process. Thus, it is likely that the ability of a cell to express class II MHC antigens in graft-rejection and graft versus host disease may confer upon it the unfortunate consequence of being the eventual target of the rejection process.

Recently, it has been proposed that the persistent expression of Ia antigens in the lentivirus-infection system may lead to lymphoproliferative disease. In this system,

Kennedy et al. (1985) have suggested that an IFN induced by lentivirus infection of peripheral blood mononuclear cells (LV-IFN) is responsible for the persistent expression of Ia antigens on macrophages, but may also play a role in restricting viral replication. A possible outcome of such a scenario in the lymph node could result in chronic lymphadenopathy due to the activation and proliferation of T cells (and possibly B cells) in this reactive environment.

DOWN-REGULATION OF CLASS II MHC ANTIGEN EXPRESSION

In addition to approaches which employ serological reagents, such as anti-Ia, anti-IFN-y, and anti-L3T4 antibodies described above, there is active investigation into pharmacological approaches that may be effective in the treatment of autoimmune diseases. Candidates for these approaches include agents which have been shown to down-regulate the expression of Ia antigen in vitro. These include: prostaglandins of the E series (Snyder et al., 1982; Kelley and Roths, 1982), analogs of adenosine 3'-5'-cyclic monophosphate (Yem and Parmely, 1981; Steeg et al., 1982b; Hanaumi et al., 1984), lipopolysaccharide (Yem and Parmely, 1981; Steeg et al., 1982b; Koerner et al., 1987; Vermeulen et al., 1987), immune complexes (Hanaumi et al., 1984; Virgin et al., 1985), α-fetoprotein (Lu et al., 1984), serotonin (Sternberg et al., 1986), and norepinephrine (Frohman et al., 1988). In addition, previous work has demonstrated that interferon-alpha/beta (IFN-α/β) and glucocorticoids [such as dexamethasone (DEX)] are also effective antagonists of IFN-y-induced cell surface Ia antigen (Ling et al., 1985; Inaba et al., 1986; Snyder and Unanue, 1982; Warren and Vogel, 1985b). These latter two classes of antagonists are of particular interest as potential therapies in the treatment of autoimmune diseases and graft rejection because: (i) they are synthesized endogenously during inflammatory processes (Gresser, 1961; Roberts et al., 1979; Shek and Sabiston, 1983; Besedovsky et al., 1975;

1986) and (ii) their production may represent a natural mechanism by which Ia antigen expression is controlled, as well as a defense against self-immunoreactivity. Given the potential role of the inducers and the antagonists of Ia antigen expression in disease states and in normal inflammatory environments, it was of interest to study the mechanisms which regulate the induction and down-regulation of Ia antigen expression. Thus, the focus of this dissertation includes an analysis of the molecular mechanisms involved in the induction of Ia antigen expression by IFN- α and its antagonism by IFN- α and DEX.

MATERIALS AND METHODS

GENERAL METHODS

Reagents. Recombinant murine IFN- γ (rIFN- γ , specific activity > 1.3 x 10⁷ U/mg) was generously provided by Genentech, Inc. (South San Francisco, CA). IFN- α / β (specific activity $\geq 5 \times 10^8$ U/mg) was the kind gift of Dr. M. Paucker (Medical College of Pennsylvania, Philadelphia, PA). The activity of each preparation for each experiment [International Units per ml (U/ml)] was confirmed using a modification of a standard antiviral assay (Rubinstein et al., 1981) in which each preparation was compared in activity to the NIH Murine Interferon-α/β Standard (Reference reagent no. G002-904-511; Vogel et al., 1982). The ORA cell line (a BALB/c-derived, constitutively Ia-positive cell line; Rosenson et al., 1981) was the source of Ia-specific mRNA for use as a positive control in Northern blots and was provided by Dr. Carol L. Reinisch (Tufts University School of Veterinary Medicine, Boston, MA). All restriction endonucleases were purchased from Bethesda Research Laboratories (Gaithersburg, MD). The synthetic progesterone derivative, R5020, was purchased from DuPont NEN Research Products (Boston, MA). Unless specifically indicated, all other chemicals used were of highest reagent grade and were purchased from Sigma Chemical Co. (St. Louis, MO). To avoid any external ribonuclease contamination, all solutions that were incapable of being autoclaved were prepared using baked glassware and sterile, irrigation-grade bottled water (H₂O; Travenol Laboratories, Inc., Deerfield, IL). Solutions capable of being autoclaved were treated with 0.1% diethylpyrocarbonate for 12 to 16 hr and were then autoclaved on a liquid cycle for 25 min. All solutions were filter-sterilized using a 0.2 µm filter (Nalge Co., Herndon, VA) to mimimize particulate contamination. Sterile, disposable plasticware was used for the containment of all filtered solutions.

Cell Culture and Treatment Protocols. Peritoneal exudate cells from thioglycollate-injected, 5-6 week old, female C3H/HeJ and C3H/OuJ mice (Jackson Laboratory, Bar Harbor, ME) were used in this study as a source of macrophages. Peritoneal exudate cells were harvested by peritoneal lavage four to five days following intraperitoneal injection of 3 ml of sterile 3% fluid thioglycollate (BBL Microbiology Systems, Cockeysville, MD). These cells (approximately 85% macrophages) were cultured in either 96-well plates (Falcon, Becton-Dickinson, Oxnard, CA) at a concentration of 2 x 105 cells/well or in 6-well plates (Falcon) at a concentration of 4 x 106 cells/well. The culture medium used was RPMI 1640 media (M.A. Bioproducts, Walkersville, MD) supplemented with 2% fetal calf serum (FCS; Hyclone Laboratories, Inc., Logan, UT), 2 mM glutamine (GIBCO Laboratories, Grand Island, NY), 30 mM N-2-hydroxy-ethyl piperazine-N'-2-ethane sulfonic acid (HEPES; Research Organics Inc., Cleveland, OH), 0.4% sodium bicarbonate, 100 IU/ml penicillin (GIBCO Laboratories), and 100 µg/ml streptomycin (GIBCO Laboratories). After an overnight incubation (37°C, 5% CO₂), to allow for adherence of the macrophages, the wells were washed with the same medium and non-adherent cells were removed by gentle aspiration. The macrophages were then treated with the indicated concentrations of test substances for the indicated periods of time.

Pulse Incorporation Analysis. Incorporation of ³H-leucine into macrophage cultures was performed to determine the optimum inhibitory concentration of cycloheximide (CHX) on protein synthesis. To determine the concentration of CHX that blocked protein synthesis maximally with minimal cell toxicity, the following experiment was performed. Macrophages were plated into 96-well tissue culture dishes (2 x10⁵ cells/well) and allowed to adhere as described previously. The cells were treated for 24 hr with medium in the absence or presence of CHX (Boehringer Mannheim Biochemicals, Indianapolis, IN) over a concentration range of 0.625 to 20 μg/ml. During the last 6 hr of

the incubation period, the cells were pulsed with 1 μ Ci/well of 3 H-leucine (Amersham Corp., Arlington Heights, IL). After the pulse period, an equal volume of an ice-cold solution of 20% trichloroacetic acid (TCA) was added to each well. The proteins were precipitated on ice for 15 min at which time the lysates were harvested onto glass fiber filters using a multi-well cell harvester (Brandel, Gaithersburg, MD). The filters were rinsed with ice-cold 10% TCA followed by ice-cold ethanol (EtOH). The filters were air-dried and counted in non-aqueous scintillation fluid (Beckman Instruments, Inc., Fullerton, CA). CHX concentrations tested above were also assessed for cell toxicity (24 hr after treatment) by microscopic examination of parallel cultures following staining of cell monolayers with trypan blue (GIBCO).

ANALYSIS OF IA ANTIGEN PROTEIN EXPRESSION

Ia antigen expression on macrophages was measured using an enzyme-linked immunosorbant assay (ELISA) which has been described in detail elsewhere (Vogel et al., 1983; Warren and Vogel, 1985). Briefly, macrophages were cultured in 96-well tissue cultures dishes and were treated with the indicated test substances for 48 hr. At that time, the monolayers were washed and were then treated with an affinity-purified, monoclonal anti-Ia^k antibody, 10-2.16 (a murine anti-Ia^k; Oi et al., 1978; Fultz et al., 1982) diluted in an Earle's balanced salt solution (EBSS; GIBCO, Grand Island, NY) which contained 10% FCS. The monolayers were incubated for 45 min at 4°C with this primary antibody. Following this incubation, the primary antibody was removed, the monolayers were washed with an EBSS solution which contained 1% FCS, and a secondary, peroxidase-conjugated goat anti-mouse IgG, F(ab')₂ antibody (Cappel Laboratories, Malvern, PA), also diluted in EBSS solution which contained 10% FCS, was added. Following a second 45 min incubation at 4°C, the secondary antibody was removed and

the monolayers were washed extensively (6-7 times) with EBSS which contained 1% FCS to remove any unbound enzyme-linked antibody. Peroxidase-conjugated secondary antibody bound to anti-Ia^k primary antibody on the surface of the macrophages was measured using the colorimetric substrate o-phenylenediamine (OPD). The substrate solution (50 µg/ml OPD, 0.1 M phosphate-citric acid buffer, pH 5.0, 0.003% hydrogen peroxide) was added to each well and incubated in the dark at room temperature for 30 min. The reaction was terminated by the addition of sulfuric acid to a final concentration of 1.6 N and the absorbance was measured at 490 nm using a 96-well plate reader (EIA; Bio-Tek, Burlington, VT). The average absorbance reading measured in samples which contained only substrate buffer and acid was substracted from all sample absorbance readings. Controls included an incubation of a primary antibody with another specificity (anti-Ia^d) and the addition of secondary antibody without primary antibody to ensure that nonspecific binding of antibody to the macrophage monolayers was not occurring. These controls yielded the same absorbance values as macrophage monolayers treated with substrate only.

ANALYSIS OF IA-SPECIFIC RNA: STEADY-STATE STUDIES

Hybridization Probes. The following cDNA constructs for the indicated I-region loci were used in the steady-state RNA studies and were generously provided by Dr. Ronald N. Germain (N.I.H., Bethesda, MD). The A_{α} probe is an ~900 basepair (bp) Bst E II-Pvu II fragment of the $A_{\alpha}{}^d$ cDNA. This fragment contains the entire protein coding sequences of the A_{α} mRNA (Davis et al., 1984). The A_{β} probe is a 464 bp fragment composed of the two Pst I fragments of pI-A $_{\beta}$ -1 ($A_{\beta}{}^d$; Robinson et al.,1983). The E_{α} probe is an ~700 bp Pst I fragment of the $E_{\alpha}{}^d$ cDNA (Dr. Mark Davis, unpublished data). The dihydrofolate reductase (DHFR) probe is a plasmid that contains a 4.0 kbp Eco RI fragment of clone λ hDHFR- ψ_1 (a human DHFR-processed psuedogene construction;

Chen et al., 1982) and was kindly provided by Dr. Arthur W. Neinhuis (N.I.H., Bethesda, MD).

RNA Isolation. Total RNA was isolated according to the guanidinium-thiocyanate protocol of Chirgwin et al. (1979). Briefly, each 6-well culture plate (2.4 x 10⁷ macrophages) was placed on ice and washed twice with 2 ml each of ice-cold EBSS, pH 7.3. The macrophages were then lysed with 1 ml/well of a solution which contained 4 M guanidinium-thiocyanate (Fluka Chemical Corp., Hauppauge, NY), 25 mM sodium citrate, pH 7.0, 0.1 M 2-mercaptoethanol (2-ME; J. T. Baker Chemical Co., Phillipsburg, NJ), and 0.5% sodium N-lauroylsarcosine. Each well was then rinsed with an additional 0.5 ml of the solution described above. The lysates were vortexed vigorously and cellular DNA was sheared by passage through a 22-gauge needle. The lysate was layered onto a 4 ml cushion of 5.7 M cesium chloride (CsCl; Bethesda Research Laboratories) which contained 25 mM sodium citrate, pH 5.0 in a 14 x 89 mm polyallomer ultracentrifuge tube (Sarstedt, Princeton, NJ) and was fractionated by centrifugation at 208,000 x g for 18 hr at 20°C in a SW41 rotor (Beckman Instruments, Inc.). The solution was decanted and the RNA pellet was air-dried. The RNA was suspended in 1 ml H2O, adjusted to a final concentration of 0.25 M sodium acetate (NaAc), pH 4.8, and was precipitated overnight at -20°C with 2.5 volumes of 95% EtOH. The RNA was recovered by centrifugation at 12,000 x g for 15 min at 4°C and the resulting pellet was resuspended in 100 µl of H2O.

Cytoplasmic RNA was isolated according to the procedure described by Mushinski et al. (1980). Briefly, 2 wells of cells (8 x 10⁶ macrophages) were placed on ice and washed twice with ice-cold EBSS which contained the ribonuclease inhibitor, heparin (1 mg/ml) (Elkins-Sinn, Inc., Cherry Hill, NJ). One ml of the ice-cold EBSS-heparin solution was added per well and the cells were removed by gentle and unidirectional scraping using disposable cell scrapers (Costar, Cambridge, MA). The cells were centrifuged at 500 x g for 10 min at 4°C and the resultant cell pellet was resuspended

in 1 ml of an ice-cold isotonic solution which contained 0.14 M sodium chloride (NaCl), 0.01 M tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl), pH 8.0, 1.5 mM magensium chloride (MgCl₂), 2 mg/ml heparin, and 250 µg/ml spermidine. The cells were next lysed on ice for 5 min by the addition of Nonidet P-40 (NP-40) to a final concentration of 0.2%. The intact nuclei were cleared from the lysate by centrifugation at 3000 x g for 10 min at 4°C. The resulting cytoplasmic supernatant was adjusted to 0.1% sodium dodecyl sulfate (SDS) and 0.01 M ethylenediamine-tetracetic acid (EDTA) and was extracted sequentially with buffer-saturated phenol (Bethesda Research Laboratories), phenol:chloroform (1:1, v/v), and chloroform. The organic and aqueous phases were separated by centrifugation at 3000 x g for 5 min at 4°C. Following the final chloroform extraction, the RNA solution was adjusted to a concentration of 0.3 M NaCl and 0.1 M MgCl₂ and the RNA was precipitated overnight at -20°C with 2.5 volumes of 95% EtOH. The RNA was recovered and resuspended as indicated above.

Nuclear RNA was isolated using a combination of both procedures decribed above. Briefly, three 6-well culture plates (6.4 x 10⁷ macrophages) were washed and treated identically to that described above in the cytoplasmic isolation procedure. Following centrifugation of the nuclei after NP-40 solubilization, the cytoplasmic supernatant was carefully decanted and the remaining nuclear pellet was saved for further purification. The nuclei were resuspended in 1 ml of guanidinium-thiocyanate solution described previously and were further solubilized by vigorous passage through a 22-gauge needle. The resulting nuclear lysate was layered onto a 0.8 ml cushion of 5.7 M CsCl in an 11 x 34 mm polyallomer ultracentrifuge tube (Beckman Instuments, Inc.) and fractionated by centrifugation in a Beckman TL-100 ultracentrifuge at 200,000 x g for 16 hr at 20°C. Following centrifugation, the solution was decanted and the RNA pellet was air-dried. The RNA was suspended identically to that described for total RNA isolation.

Total RNA, cytoplasmic RNA, and nuclear RNA preparations were quantified by measurement of absorbance at 260 nm. For Northern blot analysis, the RNA

concentration was also verified by staining the gel with ethidium bromide (EtBr) followed by visual examination of the gel under ultraviolet (UV) light (Maniatis et al., 1982). In addition to equalizing for RNA concentrations between various treatments using the absorbance value and EtBr staining, the expression of a constitutively expressed gene, dihydrofolate reductase (DHFR), was simultaneously analyzed (see below).

Electrophoresis, Blotting, Hybridization, and Detection. For Northern blot hybridization, total cellular RNA, cytoplasmic RNA, or nuclear RNA (10 - 15 μg/treatment) was denatured in loading buffer which contained 1X MOPS buffer [0.04 M morpholinopropanesulfonic acid (MOPS), pH 7.0, 10 mM NaAc, pH 4.8, and 1 mM EDTA, pH 8.0], 50% formamide (Fluka Chemical Corp.), 2.2 M formaldehyde, 2% Ficoll, 0.02 M EDTA, 0.01% xylene cyanol, and 0.01% bromphenol blue by heating for 10 min at 65°C. The RNA was electrophoresed on a 1% agarose gel which contained 0.22 M formaldehyde, 1X MOPS buffer, and 1 µg/ml EtBr in gel-running buffer (1X MOPS buffer) until the bromophenol blue dye front had migrated ~70% of the total gel distance (approximately 10 cm of a 15 cm length gel). The RNA gel was then soaked in 20X sodium chloride-sodium citrate (SSC; 1X SSC is 0.15 M NaCl, 0.015 M sodium citrate) for 1 hr at which time the gel was transferred to a UV-illumination source and photographed. The fractionated RNA was transferred overnight onto nitrocellulose paper, BA 85 (Schleicher and Schuell, Keene, NH), in 20X SSC by capillary action. For slot blot hybridization, cytoplasmic or nuclear RNA (2-10 µg/treatment) was diluted to a volume of 50 µl with H₂O. An equal volume of a solution which contained 12X SSC and 4.4 M formaldehyde was added to the RNA sample. A volume of 50 μl was applied per slot in duplicate using a Minifold Slot Blot Manifold (Schleicher and Schuell). Following sample application, 50 µl of 10X SSC was added to each slot to rinse out the slot wells.

The nitrocellulose filters were quickly immersed in 5X SSC, air-dried, and baked in a vacuum oven at 80°C for 2 hr. The filters were then prehybridized overnight at 40°C

in a buffer which contained 10% dextran sulfate (Pharmacia Inc., Piscataway, NJ), 40% formamide (Fluka Chemical Corp.), 4X SSC, 0.01 M Tris-HCl, pH 7.6, 1X Denhardt's solution [0.02% bovine serum albumin (BSA), Ficoll, and polyvinylpyrollidone each], and 0.05 mg/ml denatured salmon sperm DNA. The filters were hybridized to 2 x 106 cpm/ml of ³²P-labeled probe in the same buffer at 40°C for 18 to 24 hr. All of the I-region cDNA constructs and the DHFR plasmid were radioactively labeled using either a random hexamer priming protocol (Pharmacia Inc.) adapted from the procedure of Feinberg and Vogelstein (1983) or "nick-translated" using a nick translation procedure (Bethesda Research Laboratories) based on the original method of Rigby et al. (1977). The oligolabeling protocol involved denaturing the DNA (~100 to 200 ng) at 90°C, cooling the template DNA to 37°C, and adding the following components to initiate the synthesis reaction [dATP, dGTP, dTTP, random hexamer primer, BSA, 50 μCi [α-32P] dCTP (3000 Ci/mmole; Amersham Corp.), and the Klenow fragment of DNA polymerase I]. The synthesis reaction was incubated at room temperature for ~12 hours at which time it was terminated by the addition of a buffer which contained EDTA and SDS. The nick-translation protocol involved the limited nicking of DNA (~200 to 300 ng) in the presence of DNase I, following by the extension of the nicks by DNA polymerase I in the presence of dATP, dGTP, dTTP, BSA, and 50 µCi [\alpha-32P] dCTP (400 Ci/mmole; Amersham, Corp.). The reaction was incubated at 15°C for 60 min and terminated by the addition of an EDTA solution. For both protocols, unincorporated [α-32P] dCTP was removed using a "spun column" (Maniatis et al., 1982) of Sephadex G-50 DNA Grade (Pharmacia Inc.). Following hybridization, the filters were washed 3 times with 2X SSC, 0.1% SDS at room temperature, and then 3 times with 0.1X SSC, 0.1% SDS at 60°C.

Due to occasional high background signals on Northern blots hybridized with probes prepared using the random hexamer priming protocol, an alternate hybridization method, which was originally developed by Church and Gilbert (1984), was adopted. Briefly, Northern blot filters were prehybridized for 1 hr at 60°C in a buffer containing 1%

BSA, 7% SDS, 0.5 M sodium phosphate, pH 7.0, and 1 mM EDTA. The filters were then hybridized with 2 x 10⁶ cpm/ml of probe in the same buffer at 60°C for 18 to 24 hr. Following hybridization, the filters were washed at 60°C for 10 minutes twice with 300 ml each of a solution which contained 0.5% BSA, 5% SDS, 40 mM sodium phosphate, pH 7.0, and 1 mM EDTA, and then washed at 60°C for 10 min 3 times with 300 ml of a solution which contained 1% SDS, 40 mM sodium phosphate, pH 7.0, and 1 mM EDTA. After the final wash, the blots were air-dried and exposed to Kodak-XAR film (Eastman Kodak Co., Rochester, NY) with intensifying screens at -70°C. A densitometer (Hoefer Scientific Instruments, San Francisco, CA) was used to scan the autoradiograms and the peak areas from the recorded scans were calculated using a digitizer (Hewlett-Packard Co., Fort Collins, CO). Densitometric tracings of autoradiograms were performed in duplicate. Duplicate area tracings of a single densitometric scan of Northern or slot blot autoradiograms were all within 5% of the average tracing value. Multiple exposures of the autoradiograms, in addition to serial dilutions of the RNA, were made to ensure that the autoradiograms were measured within a linear range of exposure. To control for unequal RNA loading onto gels or slot blots, the Ia-specific signal for a given treatment was adjusted relative to its corresponding DHFR signal. The effect of various treatments on Ia expression relative to the RNA derived from control (medium-treated) macrophages was quantified using the following formula:

	(Ia _{treatment})/(DHFR _{treatment})
RATIO OF STIMULATION =	
	(Iacontrol)/(DHFRcontrol)

Unless specifically indicated, the (Ia_{control})/(DHFR_{control}) values were calculated from RNA's harvested at 24 hr from medium-treated macrophage cultures. In certain experiments where the effect of a particular treatment is shown separately for Ia mRNA and

DHFR mRNA, the digitized values which correspond to the areas under the densitometric tracings are presented without calculation of the "Ratio of Stimulation".

ESTABLISHMENT OF PROTOCOLS FOR NUCLEAR TRANSCRIPTION STUDIES

Steady-state analysis provides a measure of RNA accumulated under the influence of inductive and inhibitory agents. Nuclear transcription ("run-on") assays allow for an indirect measure of the rate at which a specific RNA species is transcribed. To monitor the efficiency and integrity of the in vitro transcription system, several parameters were first established and optimized. These included: (i) demonstration of linearity of transcript binding to specific cDNA-containing plasmids on nitrocellulose; (ii) determination of the optimal hybridization period; (iii) determination of hybridization efficiency of transcripts to cDNAs on nitrocellulose; and, (iv) demonstration of strand-specificity of transcript binding. To study the first three parameters, a radiolabeled An-transcript (homologous to A_α-specific mRNA) was prepared by synthesizing a T7-polymerase-directed transcript from pGEM-1-A $_{\alpha}$. The plasmid pGEM-1-A $_{\alpha}$ contains a full length A $_{\alpha}{}^d$ cDNA insert cloned into the Eco RI site of pGEM-1. T7-directed polymerization of the pGEM-1-Aq. construct yields transcripts of "message-sense" ("+") and SP6-directed polymerization yields transcripts of "anti-sense" ("-") RNA. The synthesis of radiolabeled, messagesense transcripts was performed according to a modified procedure described by Melton et al. (1984). pGEM-1-Aq was first linearized by digestion with Bam H1 in the presence of 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 7 mM MgCl₂, and 2 mM 2-ME at 37°C for 2 hr. The transcription mixture, which contained transcription buffer (40 mM Tris-HCl, pH 7.5, 6 mM MgCl₂, 2 mM spermidine, and 10 mM NaCl), 10 mM DTT, 1 U/µl RNasin ribonuclease inhibitor (Promega Biotec, Madison, WI), 2.5 mM each of the ribonucleotides ATP, CTP, GTP, 2.25 mM UTP, 100 μCi [5,6-3H] UTP (Amersham

Corp.), 2 µg linearized plasmid, and 20 Units T7 RNA polymerase (Bethesda Research Laboratories), was incubated for 60 min at 37°C. Following the synthesis reaction, the template DNA was digested at 37°C for 15 min by the addition of 5 Units of DNase 1-RNase free. The RNA was purified by a series of equal volume phenol:chloroform (1:1, v/v) and chloroform extractions. The aqueous phase of the last extraction was adjusted to a final concentration of 0.25 M NaAc and the RNA was precipitated with 2.5 volumes of EtOH at -20°C overnight. The RNA was recovered by centrifugation at 12,000 x g for 15 min at 4°C and resuspended in 100 µl H₂O. The RNA was precipitated in the presence of 2.5 M ammonium acetate and 2 volumes of EtOH 2 times for 30 min each at -70°C to remove any unincorporated ³H-UTP. The concentration of radiolabeled transcript synthesized was calculated using the specific activity of the isotope, the counts per min associated with a sample of the RNA, and the average molecular weight of the An-specific transcript. Approximately 10 µg of labeled RNA was synthesized per 1 µg of input template. The quantity of RNA synthesized was also verified by absorbance at 260 nm. To insure that the T7-polymerase directed transcripts were of proper length, a sample of the transcription reaction mixture was analyzed electrophoretically. Ethidium bromide staining of the gel revealed a single population of transcripts of approximately 800 nucleotides. This transcript length was consistent with that expected given the construction of the pGEM-1-A_Q plasmid (Dr. Jim Miller, personal communication).

cDNA Excess Experiments. Increasing quantities of radiolableled, message-sense RNA were mixed with a hybridization solution (200 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.6 M NaCl, 5X Denhardt's solution, 0.2% SDS,0.1% NaPPi, 250 µg/ml yeast tRNA, 100 µg/ml salmon sperm DNA) and were applied to nitrocellulose filters to which 1 µg per slot of the plasmid which contained the $A_{\alpha}{}^d$ cDNA (pCEXV- A_{α}) and 1 µg per slot of an irrelevant plasmid, pUC9 (that had been prehybridized in the same solution) were immobilized. The pCEXV- A_{α} construct contains the protein coding sequences of the

A_α^d gene and was provided by Dr. Jim Miller (N.I.H., Bethesda, MD) and the pUC9 plasmid was provided by Dr. Daniel R. Schoenberg (U.S.U.H.S., Bethesda, MD). The filters were hybridized at 40°C for 1 day at which time they were washed with 3 changes of 1X SSPE, 0.1% SDS for 30 min each at 60°C. The filters were air-dried and counted in non-aqueous scintillation fluid.

Determination of Optimal Hybridization Period. To determine the optimum length of the hybridization period, 100 ng of radiolabeled, "message-sense" RNA (T7-transcript) was hybridized to identical filters prepared for the cDNA excess study and hybridized at 40°C for varying periods of time (1 to 4 days). The filters were washed as described above and subsequently counted in non-aqueous scintillation fluid.

RNA Polymerase II Activity Determination. To determine the relative RNA polymerase II activity in nuclei isolated from macrophages, nuclei were incubated with ³H-UTP in the absence or presence of 0.5 μg/ml α-amanitin (Kedinger et al., 1970; Roeder, 1974; Darnell et al., 1986). To do this, nuclei that had been isolated from 1.0 x 10⁸ macrophages (treated with medium only for 24 hr) were elongated in vitro as described previously with the substitution of 100 μCi of [5,6-³H]-UTP (Amersham Corp.) for 250 μCi of [α-³²P]-UTP. Additionally, to one-half of the nuclei sample, α-amanitin (0.5 μg/ml) was added. Following elongation, 2% of the total volume was sampled and adjusted to final concentration of 20 mM NaPPi and 5% TCA. The mixture was incubated for 30 min on ice to precipitate the RNA. The precipitate was recovered on GF/B filters (Whatman International, Ltd., Maidstone, England) using a vacuum filtration manifold (Hoefer Scientific Instruments) and the filters were washed with a 5% TCA solution which contained 20 mM NaPPi. The washed filters were air-dried and counted in non-aqueous scintillation fluid.

Determination of Transcript Hybridization Efficiency. The efficiency of transcript hybridization to cDNAs immobilized on nitrocellulose filters was determined using the radiolabeled A_{α} -specific transcript for hybridization to nitrocellulose filters containing the cDNA clone of $A_{\alpha}^{\ d}$. One-hundred ng of the radiolabeled, "message-sense" RNA (T7-transcript) was mixed with a hybridization solution (identical to that used for the cDNA excess study) and applied to nitrocellulose filters (that had been prehybridized in the same solution) which contained 1 μ g per slot of the $A_{\alpha}^{\ d}$ cDNA (pCEXV- A_{α}) or 1 μ g per slot of an irrelevant plasmid (pUC9). Following a 3 day hybridization period, all the filters were washed with 3 changes of 1X SSPE, 0.1% SDS for 30 min each at 60°C. The filters were air-dried and counted in non-aqueous scintillation fluid. The counts recovered after washing were compared to the counts obtained from a sample of 100 ng of radiolabelled "message-sense" RNA directly applied to nitrocellulose.

Strand-Specificity Experiments. In the strand-specificity experiments, DNA derived from M13mp9 E_{β} "+" ("message-sense") and M13mp9 E_{β} "-" ("anti-sense") phage constructs were applied to nitrocellulose filters. Single-stranded phage stocks which contained the E_{β} cDNA insert cloned in both orientations into the Eco R1 site of M13mp9 were generously provided by Dr. Jim Miller. These phage stocks were used to infect a fresh culture of Escherichia coli, strain JM103. Six hr post infection the cells were pelleted at 4000 x g for 10 min. Final concentrations of 5% polyethylene glycol (8000) and 0.5 M NaCl were added to the phage supernatant. Following a 30 min precipitation at room temperature, the phage were pelleted by centrifugation at 9000 x g for 15 min. The phage were resuspended in 100 μ l of TES (20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 0.1 mM EDTA) and disrupted by extraction with an equal volume of buffer-equilibrated phenol. The aqueous phase was removed, adjusted to a final concentration of 2.5 M ammonium acetate, and the DNA was precipitated overnight at -20°C with 2 volumes of EtOH. Following centrifugation at 12,000 x g for 15 min at 4°C, the phage DNA was recovered

and resuspended in 50 μl of TES. Absorbances at 260 nm were taken to determine the single-stranded DNA concentration. These single-stranded DNA molecules which contained E_β sequences were diluted in 1X SSC and 1 μg per slot was applied. The nitrocellulose filters were air-dried, baked in a vacuum oven at 80°C for 2 hr, and prehybridized overnight. Transcripts synthesized in the <u>in vitro</u> transcription elongation assay derived from medium-treated and rIFN-γ-treated (5 U/ml) nuclei preparations were hybridized to these filters for 3 days. Due to high background signals using the standard washing conditions, more stringent washing conditions were employed. Filters were washed 3 times with 2X SSPE, 0.1% SDS at room temperature, and then 3 times with 0.05X SSPE, 0.1% SDS at 60°C. After the final wash, the slot blots were air-dried and exposed to Kodak XAR-film with intensifying screens at -70°C. The average exposure time for adequate signal detection was 4 to 8 days. Densitometric tracings of the autoradiograms and area determinations of the tracings were performed as described earlier.

ANALYSIS OF IA-SPECIFIC RNA: NUCLEAR TRANSCRIPTION STUDIES

Hybridization Probes. For the nuclear transcription "run-on" experiments, $A_{\alpha}{}^d$, $A_{\beta}{}^d$, $E_{\alpha}{}^d$, and $E_{\beta}{}^d$ inserts cloned into a mammalian expression vector, pCEXV, were used (Miller and Germain, 1986). These were provided by Dr. Jim Miller and they contain the entire protein coding sequences of the indicated I-region genes. The DHFR probe was identical to that used in the steady-state RNA studies. The plasmid, pSP64, was generously provided by Dr. Daniel R. Schoenberg.

Nuclei Isolation and Purification for Transcription Studies. Nuclei were isolated according to a modification of the procedure described by Martin et al. (1986). Four to six 6-well culture plates (approximately 1×10^8 to 1.5×10^8 macrophages) per treatment were

placed on ice and washed twice with ice-cold EBSS. The macrophages were then scraped into ice-cold EBSS (1ml/well) and the suspension was centrifuged at 500 x g for 10 min at 4°C. The cell pellet was resuspended in 0.3 ml of 0.25 M sucrose solution in Buffer A [15mM HEPES, pH 7.6, 60 mM potassium chloride (KCl), 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM ethylene glycol bis- (β-aminoethyl ether) N-N-N'-N'-tetraacetic acid (EGTA), 2 mM EDTA, 2 mM dithiothreitol (DTT)]. One and two-tenths ml of 1.4 M sucrose in Buffer A was added to the resuspended pellet and the cells were homogenized on ice with 20 strokes of a Type A (tight-fitting pestle) Dounce homogenizer (Wheaton Scientific, Millville, NJ). The homogenate was layered onto a 1 ml cushion of 1.6 M sucrose in Buffer B (15 mM HEPES, pH 7.6, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 0.1 mM EGTA, 0.1 mM EDTA, and 2 mM DTT). The nuclei were isolated by centrifugation at 100,000 x g for 1 hr at 4°C using a TL-100 ultracentifuge (Beckman Instruments, Inc.). The cytoplasmic fraction was carefully removed and the residual sucrose cushion was carefully decanted. The remaining nuclear pellet was resuspended in 1 ml of a 1.25 M sucrose solution in Buffer A and NP-40 was added to a final concentration of 0.5%. The nuclei were incubated for 5 min on ice and were further purified by centrifugation through a 1 ml cushion of 1.5 M sucrose in Buffer A using a Beckman TL-100 ultracentrifuge at 3600 x g for 15 min at 4°C. The nuclei were resuspended in 400 µl of a storage buffer which consisted of 20 mM HEPES, pH 7.6, 50% glycerol (v/v), 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, and 0.125 mM phenylmethylsulfonylfluoride. Nuclei were active transcriptionally for two months when stored in this buffer at -70°C (Martin et al., 1986).

RNA Labeling by In Vitro Transcription Elongation. Nascent RNA was labeled according to a modification of the procedure described by Martin et al. (1986). A 400 µl sample of purified nuclei resuspended in storage buffer was mixed with an equal volume of a transcription reaction mixture [50 mM HEPES, pH 7.8, 6 mM MgCl₂, 4 mM manganese

chloride (MnCl₂), 6 mM spermidine, 10 mM DTT, 0.4 M ammonium sulfate, 1.6 mM each of the ribonucleotides ATP, CTP, GTP (Pharmacia Inc.), and 250 μCi of [α-32P] UTP (400 Ci/mmol; Amersham Corp.). This mixture was incubated for 45 min at 32°C in a shaking water bath. The transcription reaction was terminated by the addition of 50 μg/ml DNase I-RNase free (Boehringer Mannheim Biochemicals). The DNase reaction was carried out for 30 min at 32°C in the presence of 2 mM calcium chloride (CaCl2) and 100 μg/ml yeast tRNA (Boehringer Mannheim Biochemicals). This mixture was deproteinized with 100 µg/ml Proteinase K (Boehringer Manneheim Biochemicals) for 45 min at 37°C in the presence of 1.5 mM EDTA and 0.1% SDS (v/v). NaCl was added to a final concentration of 100 mM and the samples were subsequently extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v). Final concentrations of 5% TCA and 20 mM sodium pyrophosphate (NaPPi) were added to the aqueous phase and the mixture was incubated for 30 min on ice to precipitate the RNA. The precipitate was harvested on a Type HA-0.45 µ, 25-mm nitrocellulose disc (Millipore Products Division, Bedford, MA) using a Type VFM1 vacuum manifold (Amicon Corp., Danvers, MA). The filters were washed extensively with 100 ml each of an ice-cold solution which contained 3% TCA and 20 mM NaPPi. The filter discs were transferred to a 6-well culture plate and any residual DNA was removed by digestion for 45 min at 37°C with 20 µg of DNase I-RNase free in 1 ml of DNase buffer (10 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 10 mM CaCl2). The filter discs were inverted and transferred to fresh 6-well cultures plates and the RNA was eluted by incubation in 1.5 ml 10X SET (1X SET; 0.01 M Tris-HCl, pH 7.6, 5 mM EDTA, 1% SDS) for 25 min at 65°C. The solution was removed and the filters were re-eluted in 1.5 ml of 1X SET for 25 min at 65°C. The two elution solutions were pooled and was adjusted to a final concentration of 125 mM NaCl. The solution was treated with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v). Thirty µg of yeast tRNA, 180 mM NaAc, and 2.5 volumes of EtOH were added to the aqueous phase. The RNA was precipitated by an overnight incubation at -20°C and

recovered by centrifugation at 9000 x g for 30 min at 4°C. The RNA was resuspended in 300 µl of a solution which contained 20 mM NaAc, pH 4.8 and 10 mM EDTA.

Detection of Specific Transcripts Among Labeled RNAs. Transcripts synthesized in the transcription elongation assay were detected by hybridization to specific cDNAs that had been applied to nitrocellulose filters using a slot blot manifold. Plasmids which contained the cDNAs for I-region loci (pCEXV-Aa, pCEXV-AB, pCEXV-Ea, and pCEXV-EB) and the DHFR clone (pJC201) were linearized with the appropriate restriction endonucleases (described below). The plasmid, pSP64 was also linearized and was included as a control for nonspecific binding. The I-region plasmids were digested with Cla 1 in the presence of 50 mM Tris-HCl, pH 8.0 and 10 mM MgCl₂. The plasmid pJC201 was digested with <u>Bgl II</u> in the presence of 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, and 1 mM DTT. The plasmid, pSP64 was digested with Eco RI in the presence of 100 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 10 mM MgCl₂. Enough DNA for 1 µg of each I-region locus per slot, 1 µg of pJC201 per slot, and 1 µg of pSP64 per slot were digested under conditions that resulted in complete digestion with 1 Unit of restriction endonuclease per µg of plasmid DNA. Digestions were carried out for 2 to 3 hr at 37°C. To ensure that linearization was complete, a small sample (1 µl) of each reaction was removed and diluted to a final concentration of 1 X TBE (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA), 2% Ficoll (Type 400-DL), 0.02 M EDTA, 0.01% xylene cyanol, and 0.01% bromophenol blue. The DNA was electrophoresed on a 0.8% agarose gel containing 1X TBE and 1 mg/ml EtBr in gel-running buffer (1X TBE). The DNA was visualized with an UV-illumination source and digestion was assessed with respect to its completeness. Following digestion, the linearized plasmids were denatured and applied to nitrocellulose filters as described by Kafatos et al. (1979). Briefly, the DNA was denatured in 0.3 M sodium hydroxide (NaOH) by heating at 65°C for 20 min. The DNA solution was then chilled on ice and neutralized with an equal volume of 2 M

ammonium acetate. Fifty-µl samples which contained either 1 µg each of the 4 I-region loci, 1 µg of pJC201 (DHFR), or 1 µg of pSP64 were applied to nitrocellulose filters (that had been previously hydrated) using a Minifold Slot Blot Manifold. Following sample application, 50 µl of 10X SCC was added to each slot to rinse out the slot wells. The nitrocellulose filters were air-dried and then baked in a vacuum oven at 80°C for 2 hr. The filters were then prehybridized in a solution which contained 50% formamide, 5X Denhardt's solution, 5X SSPE (1X SSPE is 0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, 1 mM EDTA), 0.1% SDS, and 200 µg/ml yeast tRNA.

The transcripts synthesized in the transcription elongation assay were denatured by heating at 90°C for 10 min and were cooled subsequently by the addition of 5 volumes (1.5 ml) of hybridization buffer [45% formamide, 4.5X SSPE, 4.5X Denhardt's solutions, 10% dextran sulfate (w/v), and 200 μg/ml yeast tRNA]. The transcript-hybridization mixture was added to the prehybridized nitrocellulose filters which contained the immobilized cDNAs. Hybridizations were carried out at 40°C for 3 days.

Following hybridization, the filters were washed 3 times (15 min per wash) with 2X SSPE, 0.1% SDS at room temperature and then washed with 3 changes of 0.1X SSPE, 0.1% SDS for 10 min at 60°C. Autoradiogrpahy was performed as described above.

The effect of various treatments on rates of Ia gene transcription relative to basal rates of Ia gene transcription from medium-treated macrophages was quantified identically to the steady-state analysis using the "Ratio of Stimulation" formula. A flow chart of the major steps in the nuclear transcription "run-on" assay is shown in Figure 2.

Statistics. Where indicated, results were compared using an unpaired Student's t-test (Snedecor and Cochran, 1980) and a difference was considered significant if it were less than p = 0.05.

Figure 2. Flow chart of the nuclear transcription "run-on" assay. The following flow chart briefly summarizes the major steps in the nuclear "run-on" transcription protocol. The detailed procedures of these steps are presented in Materials and Methods.

Nuclear Transcription "Run-on" Assay

Culture and treat macrophages			
*			
Isolate macrophage nuclei			
+			
Incubate nuclei with ³² P-UTP, ATP, CTP, GTP, and reaction mixtu	ıre		
Elongate pre-initia	ated transcripts	3	
Digest with DNase and proteinase K			
Isolate radiolabeled nuclear transcrip	ts		
↓			
Hybridize to filter which contains cDNA's	I-region		1
	DHFR		Pre-hybridization
	pSP64	***************************************	
Wash filters and autoradiograph			1
1	I-region	_	
1	DHFR		Post-hybridization
Y	pSP64		
Quantify by scanning densitometry			1

RESULTS

PROTEIN ANALYSIS OF INDUCTION OF IA ANTIGEN EXPRESSION BY rIFN-γ AND ITS DOWN-REGULATION BY IFN-α/β AND DEX

Ia antigen expression has been shown to be induced by natural and recombinant IFN-γ in a variety of murine systems (Steeg et al., 1982a; Paulnock-King et al., 1983; Warren and Vogel, 1985a; Warren and Vogel, 1985b). Warren and Vogel (1985b) demonstrated that rIFN-y induced Ia antigen expression in a dose-dependent fashion in thioglycollate-elicited C3H/HeJ peritoneal macrophages. By 48 hr, in the presence of 5.0 -10.0 U/ml rIFN-γ, Ia antigen expression was induced maximally (as measured by an ELISA assay or an antibody and complement-mediated cytotoxicity assay). Using an identical induction system, Warren and Vogel (1985b) and Ling et al. (1985) examined the effects of the synthetic glucocorticoid, dexamethasone (DEX), and IFN- α/β , on rIFN-y-induced Ia antigen expression. Simultaneous treatment of macrophages with DEX and rIFN-y resulted in antagonism of induced Ia expression at a protein level. Concentrations of 1 x 10⁻⁹M - 1 x 10⁻⁴M DEX were found to down-regulate Ia antigen expression induced by 5 - 10 U/ml rIFN-y in a dose-dependent manner. In addition, higher concentrations of DEX (1 x 10⁻⁶M - 1 x 10⁻⁴M) also reduced basal levels of Ia antigen significantly. Other glucocorticoids (both naturally-occurring and synthetic), including hydrocortisone, corticosterone, and triamcinolone acetonide, were also found to reduce levels of Ia antigen induced by rIFN-γ. In contrast, treatment of macrophages with non-glucocorticoid steroid hormones, including progesterone and dihydrotestosterone, had no effect on rIFN-γ-induced levels of Ia antigen. In the study by Ling et al. (1985), basal levels of Ia antigen expression were unaltered following prior or simultaneous treatment with as much as 100 U/ml IFN-α/β. Down-regulation of Ia antigen by IFN-α/β (100

U/ml) was observed both at optimally-induced levels of Ia antigen (i.e., at 5.0 U/ml rIFN- γ) and sub-optimally-induced levels of Ia antigen (i.e., at 0.5 U/ml rIFN- γ). The antagonistic effect of IFN- α / β on rIFN- γ -induced Ia antigen expression was also dose-dependent: 100 U/ml IFN- α / β was more effective than 10 U/ml IFN- α / β in down-regulating Ia antigen expression induced by optimal (5.0 U/ml) or suboptimal (0.5 U/ml) concentrations of rIFN- γ .

The data in Table 1 corroborate these findings for both the induction of Ia antigen expression by rIFN-γ as well as and the antagonism of rIFN-γ-induced Ia antigen expression by IFN-α/β and DEX. By 48 hr in culture in the presence of 5.0 U/ml rIFN-γ, Ia antigen expression (as detected by ELISA) was increased significantly. Simultaneous treatment of cultures with rIFN- γ and either IFN- α/β (100 U/ml) or DEX (1 x 10⁻⁵M) caused a significant reduction in levels of Ia antigen expression. Since DEX has been shown to bind both glucocorticoid and progesterone receptors (Lippman et al., 1977; Eisen et al., 1981), the synthetic progestin, R5020, which binds specifically to the progesterone receptor (Philibert and Raynaud, 1973), was tested for its effects on rIFN-γ-induced Ia antigen expression. The absence of any effect with this drug strongly supports the hypothesis that the effects of DEX are mediated through the glucocorticoid receptor and are not due to the progesterone receptor-binding capacity which has been associated with DEX. The data presented in Table 1 illustrate that R5020 had no effect on either basal or rIFN-γ-induced levels of Ia antigen expression over a dose range of 1 x 10⁻⁷M - 1 x 10⁻⁵M. These findings support and extend earlier observations which demonstrate the glucocorticoid specificity of the antagonism (Warren and Vogel, 1985b).

These confirmations and extensions of previous studies on Ia antigen induction and down-regulation at a <u>protein level</u> provided the background for the direction of study of this dissertation. The following experiments focus on a molecular analysis of the mechanisms which underlie the induction of Ia antigen expression by rIFN- γ and its down-regulation by IFN- α/β and DEX. The next series of experiments was designed to

TABLE 1

Effect of IFN-α/β, DEX, and R5020 on Basal and rIFN-γ-Induced Levels of Ia Antigen

A 1.11cl	Ass. Later	Treatment	
Additive	Concentration	Medium	rIFN-γ (5.0 U/ml)
Medium only	,	0.247 ± 0.027^{b}	0.650 ± 0.043
IFN-α/β	100 U/ml	0.309 ± 0.043	0.474 ± 0.069 ^c
DEX	1 x 10 ⁻⁵ M	0.202 ± 0.046	0.438 ± 0.068 ^c
R5020	1 x 10 ⁻⁷ M	0.225 ± 0.030	0.612 ± 0.078
	$1 \times 10^{-6} M$	0.259 ± 0.010	0.614 ± 0.043
	1 x 10 ⁻⁵ M	0.250 ± 0.016	0.580 ± 0.052

^aMacrophage cultures were treated with the indicated concentrations of medium or rIFN- γ and/or IFN- α/β , DEX, or R5020 for 48 hr and then assayed for Ia antigen expression by ELISA.

bThe results are expressed as the arithmetic means ± standard error of the mean (S.E.M.) of the absorbance of three independent experiments with 6 - 8 replicates per treatment per experiment.

^CDiffered significantly (p < 0.05) from treatment with rIFN- γ only in an unpaired Student's *t*-test.

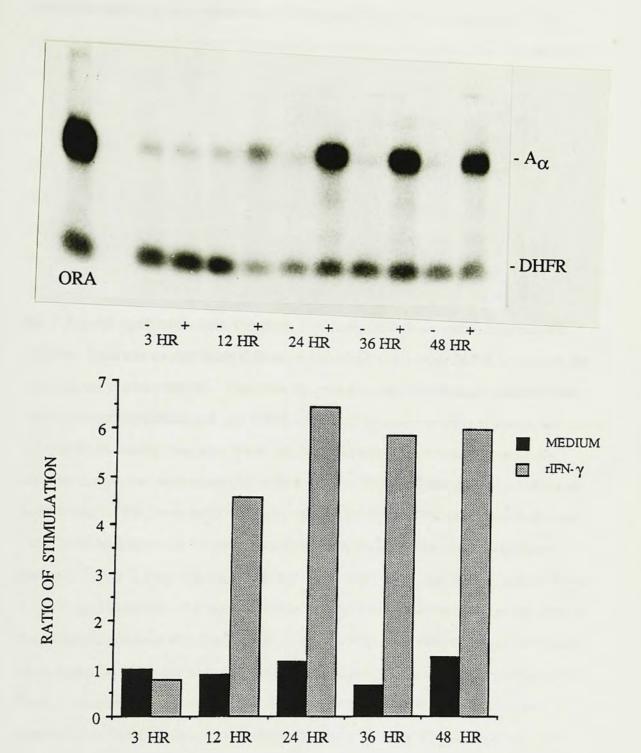
determine if the induction of Ia protein expression by rIFN- γ and the down-regulation of rIFN- γ -induced Ia protein expression by IFN- α/β and DEX were mediated by cognate changes in levels of Ia-specific mRNA.

STEADY-STATE ANALYSIS OF IA-SPECIFIC RNA

To study the regulation of I region gene expression in primary murine macrophages, methods for the isolation, fractionation, and detection of Ia-specific RNA first had to be developed. This involved: (i) establishment of cell culture conditions that were compatible with RNA isolation techniques (i.e., plating, treatment, and harvesting procedures); (ii) optimization of methods for the isolation of total RNA, cytoplasmic RNA, and nuclear RNA, such that degradation of RNA was minimized and yields of RNA were adequate for detection and analysis of specific RNA species; (iii) optimization of methods for the fractionation and the transfer of RNA to nitrocellulose membranes, as well as for the direct sample application to nitrocellulose membranes; (iv) identification of a suitable internal control (DHFR) to monitor differences in RNA that might result from harvesting or sample application; and, (v) determination of optimal inhibitory doses of specific metabolic inhibitors for the characterization of their effects on levels of steady-state RNA. The methods that were established are detailed in the Materials and Methods section. The rationale for using a particular procedure will be discussed in the context of specific experiments.

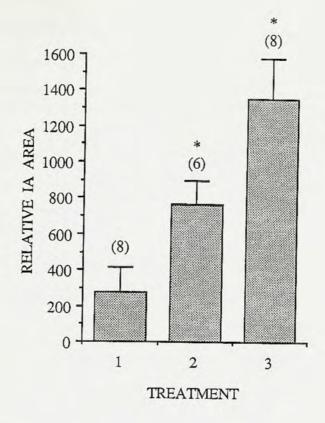
Kinetics of induction of A_{α} -specific mRNA with rIFN- γ . The accumulation of total cellular A_{α} -specific RNA in C3H/HeJ murine macrophages treated with 5.0 U/ml rIFN- γ was first examined by Northern blot analysis and the results are illustrated in Figure 3. This dosage of rIFN- γ was chosen since it was demonstrated in previous studies

Figure 3. Kinetics of the induction of A_{α} -specific total mRNA by rIFN- γ . Macrophages were cultured for the indicated periods of time in the absence or presence of rIFN- γ (5.0 U/ml). At the indicated times, total RNA was extracted, electrophoresed, and transferred to mitrocellulose (Northern blot), and analyzed using the A_{α} and DHFR cDNA probes as described in Materials and Methods. The top panel is a Northern blot and the bottom panel is a quantitative representation of the data derived from densitometric scan of the blot with each time point positioned below its corresponding lane on the blot. In addition, the lanes on the blot are labeled with a "-" or a "+" which indicates treatment with medium or IFN- γ , respectively. The Ratio of Stimulation (bottom panel) was determined as described in Materials and Methods from duplicate area tracings of a single densitometric scan of the Northern blot shown in the top panel. RNA extracted from the constitutively Ia-positive, macrophage cell-line, ORA, was included in the Northern blot as a positive control.



to result in optimal protein expression (Warren and Vogel, 1985b; Vogel et al., 1986). Inspection of a typical Northern blot autoradiogram (top panel) showed that sub-maximal steady-state levels of A_{α} -specific mRNA are detected by 12 hr and maximum levels by 24 hr after IFN- γ treatment. In addition to being hybridized with an A_{α} -specific radiolabeled cDNA sequence, the RNA was also hybridized with a DHFR-specific radiolabeled cDNA. The DHFR signal served as an internal control since its expression was unchanged by rIFN-γ treatment (Figure 4). Figure 4 illustrates the relative constancy of DHFR message levels (bottom panel) in macrophage cultures treated for 24 hr with medium, 0.5 U/ml rIFN-γ, and 5.0 U/ml rIFN-γ (Treatments 1, 2, and 3, respectively) in contrast to increased levels of A_{\alpha}-specific mRNA (top panel) from the same treatment samples. Treatments 2 and 3 differed significantly from Treatment 1 with respect to their relative Ia expression; however, there was no significant difference with respect to relative DHFR expression (for p values, see Figure 4 legend). Therefore, by comparing the DHFR signal obtained from samples of different treatments and different time points, it was possible to control for unequal RNA loading onto gels. To do this, the autoradiogram was densitometrically scanned and the data were quantified in the form of a "Ratio of Stimulation" (see Materials and Methods). The lower panel of Figure 3 shows the Ratio of Stimulation for each time point positioned below its corresponding lane on the Northern blot. In the experiment shown in Figure 3, there was a 6.5-fold increase in steady-state, A_{\alpha}-specific mRNA levels by 24 hr post-induction. The level of this Ia-specific RNA remained high through 48 hr in the continued presence of inducer (rIFN-y). Similar time course kinetics were performed on cytoplasmic preparations to examine the accumulation of mRNA in the cytoplasm only. Table 2 illustrates that A_α-specific mRNA was first detected 6 hr post-induction with 5.0 U/ml rIFN- γ . Based on five independent time-course experiments, there was a 5.7 \pm 1.0 fold increase in A_{α} -specific mRNA by 24 hr of treatment with rIFN- γ . As was observed for the total RNA preparation, A_α-specific mRNA remained high in cytoplasmic preparations through 48 hr post-induction. These data are consistent with the previously

Figure 4. Effect of rIFN- γ treatment on the steady-state levels of A_{α} - and DHFR-specific mRNA. Cytoplasmic or total RNA was isolated from macrophages that had been treated for 24 hr with medium (Treatment 1), 0.5 U/ml rIFN- γ (Treatment 2), or 5.0 U/ml rIFN- γ (Treatment 3). The RNA preparations were either electrophoresed and transferred to nitrocellulose (Northern blot) or directly applied to nitrocellulose (slot blot) and analyzed using A_{α} and DHFR cDNA probes. For each experiment, duplicate area measurements from individual densitometric tracings of autoradiograms were taken. The number in parentheses above each treatment represents the number of individual experiments ("n") from which the average value was derived. The results represent the arithmetic means \pm S.D. of "n" experiments. The results were compared using an unpaired Student's *t*-test. An asterisk above a treatment indicates that the results differed significantly (p < 0.05) from Treatment 1 of the same graph.



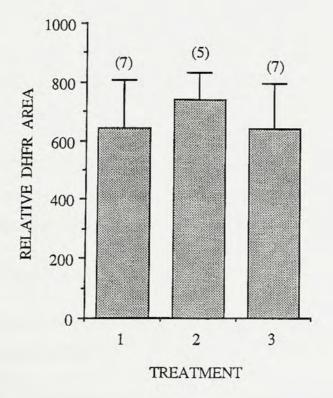


TABLE 2 $\begin{tabular}{ll} Time Course Profile of A_{α}-Specific Cytoplasmic mRNA Induced by rIFN-γ^a \\ \end{tabular}$

Hours Post-Induction	Ratio of Stimulation
0	1.0 ^b
3	$0.8 \pm 0.2^{\circ}$ (4) ^d
6	1.7 ± 0.2 (2) ^e
12	3.7 ± 1.4 (4) ^e
24	5.7 ± 1.0 (4) ^e
36	4.6 ± 1.7 (4) ^e
48	5.3 ± 1.1 (2) ^e

^aMacrophages were cultured in the absence or presence of rIFN- γ (5.0 U/ml). At the indicated times, cytoplasmic RNA was extracted and analyzed using A_{α} and DHFR cDNA probes as described in Materials and Methods.

 $b_{The\ A_{\alpha}}$ -specific mRNA isolated from macrophages cultured in medium only did not vary significantly over the time course studied. Therefore, the $Ia_{control}$ term in the denominator of the Ratio of Stimulation formula was the medium-treated, 0 hr mRNA value.

^cThe results are expressed as the arithmetic means \pm S.D.

d The number in parentheses indicates the number of independent experiments from which that particular value was derived.

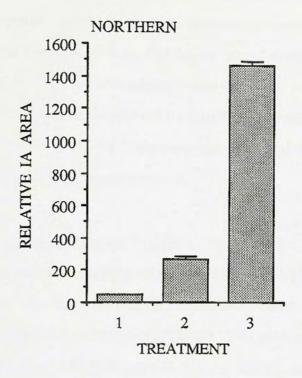
eDiffered significantly (p < 0.05) from 0 hr control as assessed by an unpaired Student's t-test.

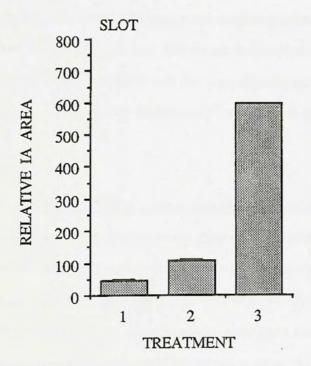
published protein kinetics which demonstrate maximum expression of Ia antigen following a 48 hr induction period with 5.0 - 10.0 U/ml natural or rIFN- γ (Warren and Vogel, 1985b; Ling et al., 1985).

Given the specificity of the cDNA probes for their respective mRNA species, an alternative, less tedious RNA quantification technique was tested. This technique allows one to apply the RNA directly on to nitrocellulose using a slot-blot manifold rather than fractionating the RNA electrophoretically prior to transfer to nitrocellulose (Northern blotting). In addition, this method allows one to process more samples than one could electrophorese on a gel under standard conditions. The data presented in Figure 5 compare these two transfer methods. Cytoplasmic preparations of RNA from macrophage cultures treated with medium, 0.5 U/ml rIFN-y, and 5.0 U/ml rIFN-y (Treatments 1, 2, and 3, respectively) were harvested. Ten µg of RNA from each treatment was subjected to electrophoresis followed by Northern blotting and 5 µg from each treatment was applied to nitrocellulose directly using a slot blot manifold. The filters were hybridized simultaneously with an A_{α} -cDNA probe and the relative A_{α} -specific signals were determined by densitometric scanning of the autoradiograms. The signal intensities observed in A_α-specific mRNA levels (top panel) with increasing doses of rIFN-γ from samples directly applied to nitrocellulose were approximately one-half that of the A_{\alpha}-specific mRNA levels from electrophoresed samples (bottom panel). This is consistent with the RNA loading per lane for electrophoresis (10 µg) compared to the application of RNA applied per slot for direct detection (5 μ g). Given the comparability of A_{α} -specific mRNA level detection between the slot blot and Northern blot methods, data collected from either method were pooled in a number of subsequent studies.

Dose-dependency of the induction of A_{α} -specific mRNA by rIFN- γ . Previous work carried out in this laboratory (Warren and Vogel, 1985a; Warren and Vogel, 1985b; Ling et al., 1985) demonstrated that rIFN- γ induced Ia protein expression in a

Figure 5. Comparison of Northern blotting and direct sample application (slot blot) methods. Cytoplasmic RNA was extracted from macrophages cultured for 24 hr in the presence of medium (Treatment 1), 0.5 U/ml rIFN- γ (Treatment 2), or 5.0 U/ml rIFN- γ (Treatment 3). For the Northern blot (top graph), 10 μ g of RNA from each treatment were electrophoresed and transferred to nitrocellulose as described in Materials and Methods. For the slot blot (bottom graph), 5 μ g of RNA from each treatment were applied directly to nitrocellulose using a slot-blot manifold as described in Materials and Methods. In this experiment, the blots were hybridized with the A_{α} cDNA probe only. The relative Ia area values represent the arithmetic means \pm S.D. of duplicate area measurements from densitometric tracings of either the Northern (top graph) or slot blot (bottom graph) autoradiograms.





dose-dependent fashion: suboptimal expression was observed at 0.5 U/ml and optimal expression at 5.0 -10.0 U/ml. To determine if these phenomena were reflected at an mRNA level, macrophage cultures were treated with varying doses of rIFN- γ and cytoplasmic RNA was harvested at 24 hr. The data in Table 3 illustrate the dependency of A_{α} -specific mRNA accumulation with respect to dose of rIFN- γ . Accumulation of A_{α} -specific RNA was sub-maximal with 0.5 U/ml rIFN- γ and maximal levels were obtained with 5.0 - 10.0 U/ml rIFN- γ . Treatment with 25.0 U/ml rIFN- γ did not further augment the accumulation of A_{α} -specific mRNA.

Induction of other I-region loci by rIFN- γ . To determine if rIFN- γ treatment resulted in increased steady-state mRNA levels of other class II MHC genes (i.e., A_{β} and E_{α}), cytoplasmic RNA was extracted from macrophages that had been treated in the absence or presence of rIFN- γ (0.5 U/ml or 5.0 U/ml). This RNA was applied directly to nitrocellulose and subsequently hybridized with A_{α} , A_{β} , and E_{α} , and DHFR cDNA probes. Table 4 demonstrates the parallel nature and dose-dependency of induction of mRNA for these three distinct I-region loci. Due to the variability in labeling efficiency of the different I-region cDNA probes inherent in this type of analysis, it was not possible to draw any conclusions about the relative expression of one I-region gene compared to another I-region gene.

Effect of the duration of rIFN- γ treatment on the induction of A_{α} -specific mRNA. To examine the effect of the length of inducer exposure on the accumulation of A_{α} -specific mRNA, cytoplasmic RNA was isolated from cultures that had been treated with rIFN- γ for varying periods of time. The RNA was applied directly to nitrocellulose and analyzed using A_{α} and DHFR cDNA probes as described in Materials and Methods. Figure 6 illustrates that the accumulation of maximal levels of steady-state, A_{α} -specific mRNA (isolated 24 hr after initial treatment) required the continued presence of inducer. Removal

Dose of rIFN-γ (U/ml)	Ratio of Stimulation		
0	1.0 ^b		
0.5	$1.7 \pm 0.4^{\circ}$ (5) ^d ,e		
5.0	3.3 ± 0.6 (5) ^e		
10.0	3.9 ± 0.9 (4)		
25.0	4.0 ± 1.8 (2)		

^aCytoplasmic RNA was extracted from macrophages cultured for 24 hr in the absence or presence of rIFN- γ (0.5, 5.0, 10.0, or 25.0 U/ml). The RNA preparations were applied to slot blots, hybridized with A_{α} and DHFR cDNA probes, and analyzed as described in Materials and Methods.

bThe Ia_{control}/DHFR_{control} term in the denominator of the Ratio of Stimulation formula was the medium-treated, 0 hr mRNA value. Therefore, the Ratio of Stimulation value for medium-treated cultures is equal to 1.0.

^cThe results are expressed as the arithmetic mean \pm S.D.

dThe number in parentheses indicates the number of independent experiments from which that particular experiment was derived.

eSignificantly different (p < 0.05) from the preceding dose of rIFN- γ , as assessed by an unpaired Student's *t*-test.

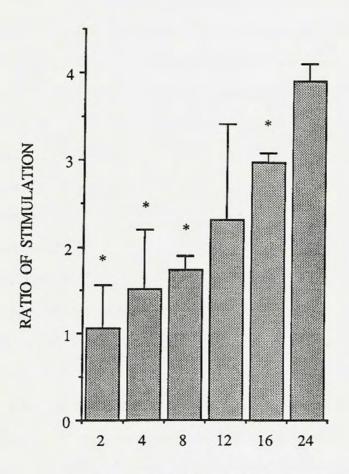
TABLE 4 Induction of A_{α} -, A_{β} -, and E_{α} -Specific mRNA by rIFN- γ^a

	RATIO OF STIMULATION ^b		
	Aα	Αβ	E_{α}
Experiment 1			
Medium	1.0	1.0	1.0
0.5 U/ml rIFN-γ	3.5	4.8	4.2
5.0 U/ml rIFN-γ	5.8	7.9	8.4
Experiment 2			
Medium	1.0	1.0	1.0
0.5 U/ml rIFN- γ	1.9	3.0	5.2
5.0 U/ml rIFN-γ	3.2	6.0	11.3

^aCytoplasmic RNA was extracted from macrophages cultured for 24 hr in the absence or presence of rIFN-γ (0.5 or 5.0 U/ml). The RNA's were applied to slot blots and hybridized to the indicated I-region cDNAs. They were subsequently analyzed by procedures described in Materials and Methods.

^bThe Ia_{control}/DHFR_{control} value in the denominator of the Ratio of Stimulation formula was the medium-treated 24 hr mRNA value from a particular hybridization with either A_{α} , A_{β} , or E_{α} . The Ratio of Stimulation values for medium-treated cultures analyzed with A_{α} , A_{β} , and E_{α} are all set equal to 1.0. Duplicate tracings were always within 5% of each other.

Figure 6. Effect of removal of rIFN- γ during the induction period on the accumulation of A_{α} -specific mRNA. Macrophage cultures were treated with 5.0 U/ml rIFN- γ . At the indicated times, the monolayers were washed three times with media and supplemented with fresh media without rIFN- γ . Cytoplasmic RNA was extracted at 24 hr after initial treatment with medium or rIFN- γ and was subsequently applied to slot blots and hybridized with A_{α} and DHFR cDNA probes. The results represent the arithmetic means \pm S.D. of 2 separate experiments. These treatments which led to a significant reduction in maximum accumulation of A_{α} -specific mRNA (p < 0.05) are indicated by the presence of an asterisk.



LENGTH OF rIFN- γ EXPOSURE (HR)

of rIFN- γ at any time during the induction period resulted in reductions in levels of steady-state, A_{α} -specific mRNA.

Effect of the protein synthesis inhibitor, cycloheximide (CHX), on the levels of <u>rIFN-y-induced A α -specific mRNA.</u> To determine if the induction of A α -specific mRNA by rIFN-γ were dependent upon the <u>de novo</u> production of a protein intermediate, A_{α} -specific mRNA levels were measured in cultures treated with rIFN- γ or rIFN- γ plus CHX, a protein synthesis inhibitor. The concentration of CHX used in this study was chosen based on the observation that 5 µg/ml CHX resulted in an 88-95% reduction in TCA-precipitable counts with no apparent toxicity to macrophages as judged by trypan blue exclusion over a 24 hr treatment period (Table 5). Macrophages were treated with rIFN-γ (5.0 U/ml) at time 0 and CHX was added at various times relative to the addition of rIFN-y. After the indicated time of CHX addition, the macrophages were incubated in the continued presence of inducer for the remainder of the 24 hr treatment period, at which time cytoplasmic RNA was isolated and assayed for A_{\alpha}-specific mRNA. Figure 7 shows the results of this experiment. In several experiments, CHX treatment alone was found to modulate DHFR message level. Therefore, only the relative areas which corresponded to the A_{α} -specific signals are shown in Figure 7. Pretreatment of the cultures with CHX 4 hr prior to rIFN-y treatment (-4 hr time point) and continued treatment during the induction period resulted in the most dramatic reduction in the accumulation of A_{α} -specific mRNA. However, the addition of CHX to cultures as late as 12 hr after exposure to rIFN-γ resulted in > 30% reduction in the levels of A_{α} -specific mRNA. These data suggest that <u>de novo</u> protein synthesis is required for the accumulation of A_α-specific mRNA.

Effect of IFN- α/β on the levels of rIFN- γ -induced A_{α} -specific mRNA. To examine whether the down-modulation of rIFN- γ -induced Ia antigen expression by IFN- α/β that was observed originally at a protein level (Ling et al., 1985), was also

TABLE 5

Determination of the CHX Concentration that Results in Optimal Inhibition

of Protein Synthesis^a

CHX Concentration	³ H-Leucine Incorporation (cpm/culture)			
(μg/ml)	Experiment 1	Experiment 2		
NONE	9619 ± 717 ^b	7716 ± 871		
0.625	NTe	949 ± 73 (88%) ^c		
1,25	1961 ± 118 (77%)	893 ± 125 (88%)		
2.5	NT	365 ± 11 (95%)		
5.0 ^d	$1029 \pm 135 \ (88\%)$	426 ± 39 (95%)		
10.0	436 ± 22 (95%)	202 ± 9 (97%)		

 a Macrophages were cultured in the absence or presence of CHX (0.625 - 10.0 μ g/ml) for 24 hr as described in Materials and Methods.

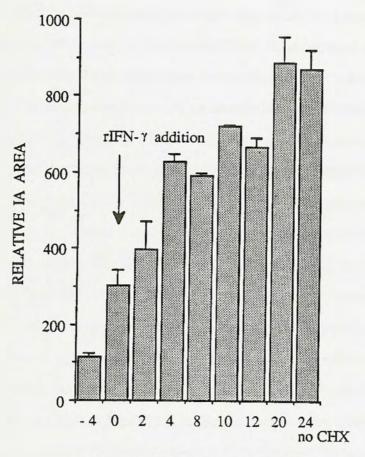
 b Results represent the arithmetic mean \pm S.E.M. of n=5 - 6 cultures.

^cPercent reduction in ³H-leucine incorporation into TCA-precipitable material compared to medium-treated cultures.

d_{This} concentration of CHX was not toxic to the cells over a 24 hr period as judged by trypan blue exclusion (98% of cells excluded trypan blue).

eNot tested.

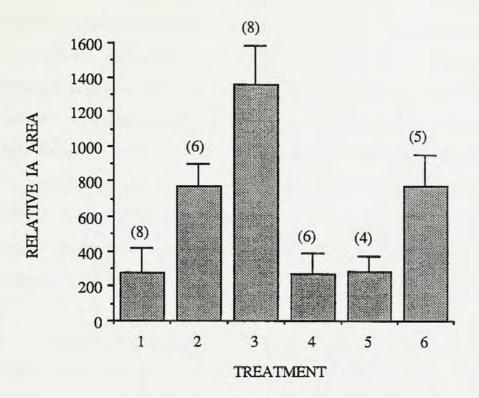
Figure 7. Effect of CHX on the accumulation of rIFN- γ -induced A_{α} -specific mRNA. At the indicated times, CHX (5 μ g/ml) was added to macrophage cultures that had been treated with rIFN- γ (5.0 U/ml) at time 0 hr. Time "-4 hr" cultures were preincubated for 4 hr with CHX prior to rIFN- γ treatment. Time "24 hr" cultures were treated with rIFN- γ alone for 24 hr. Cytoplasmic RNA was harvested 24 hr post-rIFN- γ treatment and analyzed on slot blots using the A_{α} cDNA probe. Due to fluctuations in DHFR levels as a result of CHX treatment, only the relative Ia area measurements are represented. The results represent the arithmetic means \pm S.D. of a single experiment, representative of 3 separate experiments.

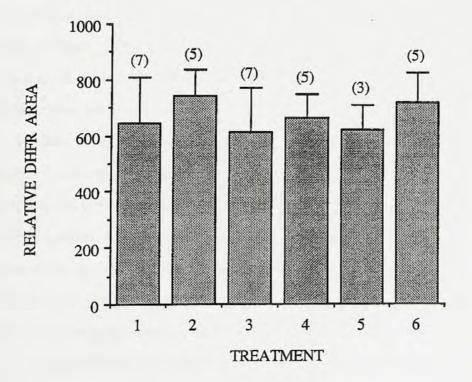


TIME OF CHX ADDITION (HR)

reflected at an RNA level, steady-state levels of A_{α} -specific mRNA were assayed after 24 hr of treatment with rIFN- γ in the absence or presence of IFN- α/β . This time point was chosen based on the kinetic data from the induction experiments which demonstrate the accumulation of maximum levels of A_α-specific mRNA 24 hr after treatment with rIFN-γ (Figure 3 and Table 2). From previous protein analysis, it was evident that IFN- α/β was an effective antagonist of rIFN-y-induced Ia antigen expression when present simultaneously with or 24 hr prior to addition of rIFN-γ. Based on these earlier observations, the following RNA experiments were performed with inducer (rIFN-y) and antagonist (IFN-α/β) present simultaneously. Although levels of DHFR-specific RNA were unaltered by rIFN-γ (Figure 4), it was also necessary to demonstrate that the levels of mRNA of this internal control were unaltered following treatment with IFN-α/β or a combination of rIFN- γ plus IFN- α/β . In Figure 8, A_{α} -specific mRNA levels (top graph) and DHFR mRNA levels (bottom graph) were measured in cytoplasmic RNA preparations of macrophage cultures treated for 24 hr with medium (Treatment 1), 0.5 U/ml rIFN-y (Treatment 2), 5.0 U/ml rIFN-γ (Treatment 3), 100 U/ml IFN-α/β (Treatment 4), 0.5 U/ml rIFN-γ plus 100 U/ml IFN-α/β (Treatment 5), and 5.0 U/ml rIFN-γ plus 100 U/ml IFN- α/β (Treatment 6). The levels of rIFN- γ -induced A_{α} -specific mRNA measured from the same samples were reduced significantly in the presence of IFN- α/β . For example, treatment with 100 U/ml IFN- α/β led to a significant reduction in the levels of A_{α} -specific mRNA induced by 0.5 or 5.0 U/ml rIFN-γ (Figure 8, top panel: Treatments 5 and 6 were found to be significantly different from Treatments 2 and 3, respectively). DHFR-specific mRNA levels from cultures treated with rIFN- γ and IFN- α/β did not differ significantly from cultures treated with rIFN-y alone (Figure 8, bottom panel: Treatments 5 and 6 were not found to be significantly different from Treatments 2 and 3, respectively). The effects of IFN-α/β on rIFN-y-induced levels of Ia-specific mRNA as represented in terms of a Ratio of Stimulation is shown in Figure 9. Figure 9 is an analysis of pooled data from densitometric profiles of Northern and slot blot autoradiograms of RNA samples from

Figure 8. Effect of rIFN- γ , IFN- α/β , and rIFN- γ plus IFN- α/β treatment on the steady-state levels of A_{α} - and DHFR-specific mRNA. Cytoplasmic or total RNA was isolated from macrophages treated for 24 hr with medium (Treatment 1), 0.5 U/ml rIFN- γ (Treatment 2), 5.0 U/ml rIFN- γ (Treatment 3), 100 U/ml IFN- α/β (Treatment 4), 0.5 U/ml rIFN- γ plus 100 U/ml IFN- α/β (Treatment 5), or 5.0 U/ml rIFN- γ plus 100 U/ml IFN- α/β (Treatment 6). The RNA preparations were either electrophoresed and transferred to nitrocellulose or applied directly to nitrocellulose and analyzed using A_{α} and DHFR cDNA probes. The number in parentheses above each treatment represents the number of individual experiments ("n") from which the average value was derived. The results represent the arithmetic means \pm S.D. of "n" experiments. The results were compared using an unpaired Student's *t*-test. With respect to relative Ia area (top graph), Treatments 5 and 6 differed significantly (p < 0.05) from Treatments 2 and 3, respectively. With respect to relative DHFR area (bottom graph), Treatments 5 and 6 did not differ significantly from Treatments 2 and 3, respectively.

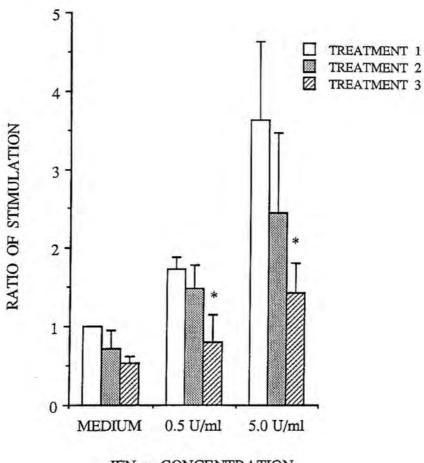




cultures treated with rIFN- γ and/or IFN- α/β . From the data, it is clear that simultaneous addition of 100 U/ml IFN- α/β to concentrations of rIFN- γ that alone induce suboptimal (0.5 U/ml) and optimal (5.0 U/ml) levels of A_{α} -specific mRNA resulted in a significant reduction in steady-state levels of A_{α} -specific mRNA (as compared to rIFN- γ -treated cultures). As had been previously reported at a protein level, the inhibition was dose-dependent: 100 U/ml IFN- α/β is a more effective antagonist of rIFN- γ -induced A_{α} -specific mRNA than 10 U/ml IFN- α/β . In addition, Northern blot analysis (Figure 10) of cytoplasmic RNA samples treated with rIFN- γ and/or IFN- α/β failed to demonstrate any smaller species of A_{α} -specific RNA, unique to IFN- α/β -treated samples, which might suggest any degradation of the A_{α} -specific mRNA in cultures treated with the IFN- α/β .

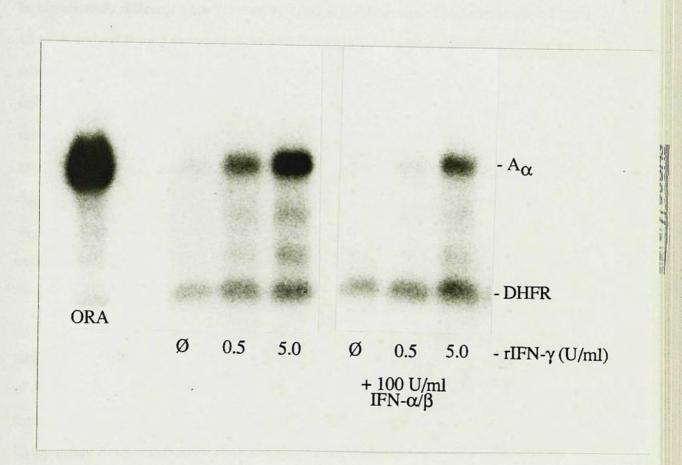
Effect of DEX on the levels of rIFN-y-induced An-specific mRNA. To determine if the glucocorticoid-mediated antagonism of rIFN-y-induced Ia antigen expression that had been demonstrated previously at a protein level were also paralleled by changes in levels of A_{α} -specific mRNA, steady-state levels of A_{α} -specific mRNA were assayed after 24 hr of treatment with rIFN-γ in the presence or absence of DEX. Warren and Vogel (1985b) demonstrated that DEX was an effective antagonist of rIFN-γ-induced Ia antigen expression when added simultaneously with rIFN-γ or 2 hr after the addition of rIFN-γ. From these observations, the following RNA experiments were performed with inducer (rIFN-γ) and antagonist (DEX) present simultaneously. The concentration of 1 x 10⁻⁵M was selected because it was shown previously to result in maximal inhibition of rIFN-γ-induced Ia protein expression (Table 1). The usefulness of DHFR as an internal control was again re-evaluated in the presence of DEX and combinations of DEX and rIFN- γ . In Figure 11, A_{α} -specific mRNA levels (top graph) and DHFR-specific mRNA levels (bottom graph) were measured in cytoplasmic RNA preparations of cultures treated with medium (Treatment 1), 0.5 U/ml rIFN-γ (Treatment 2), 5.0 U/ml rIFN-γ (Treatment 3), 1 x 10^{-5} M DEX (Treatment 4), 0.5 U/ml rIFN- γ plus 1 x 10^{-5} M DEX (Treatment 5),

Figure 9. Effect of IFN-α/β on the steady-state levels of rIFN- γ -induced A_{α} -specific mRNA. Cytoplasmic RNA was harvested from macrophage cultures treated for 24 hr with medium or rIFN- γ (0.5 or 5.0 U/ml) in the absence of IFN-α/β (Treatment 1), in the presence of 10 U/ml IFN-α/β (Treatment 2), or in the presence of 100 U/ml IFN-α/β (Treatment 3). The data were derived from both Northern and slot blots that had been analyzed using A_{α} and DHFR cDNA probes as described in Materials and Methods. The results represent the arithmetic mean \pm S.D. of 3 separate experiments. The results were compared using an unpaired Student's *t*-test. An asterisk indicates that cultures treated with rIFN- γ (either 0.5 or 5.0 U/ml) and 100 U/ml IFN-α/β differed significantly (p < 0.05) from cultures treated with rIFN- γ (either 0.5 or 5.0 U/ml).



rIFN- γ CONCENTRATION

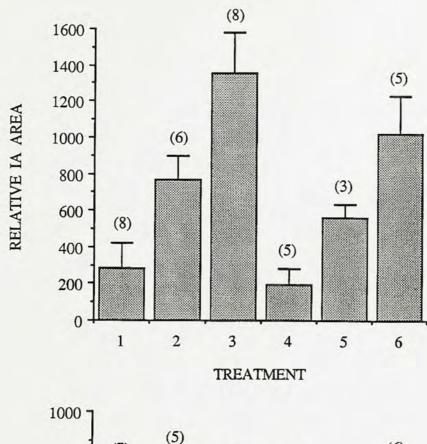
Figure 10. Autoradiogram of Northern blot which illustrates the effect of IFN- α/β on the steady-state levels of rIFN- γ -induced A_{α} -specific mRNA. Cytoplasmic RNA was harvested from macrophage cultures that had been treated for 24 hr with medium, rIFN- γ (0.5 or 5.0 U/ml), IFN- α/β (100 U/ml), or combinations of rIFN- γ and IFN- α/β . The RNAs were then analyzed for A_{α} - and DHFR-specific mRNA as described in Materials and Methods.



and 5.0 U/ml rIFN- γ plus 1 x 10⁻⁵M DEX (Treatment 6). As was observed previously (Figures 4 and 8), the levels of rIFN- γ -induced A_{α} -specific mRNA (Figure 11, top panel) were significantly altered with treatment of DEX (Treatments 5 and 6 were found to be significantly different from Treatments 2 and 3, respectively). DHFR-specific mRNA levels from cultures treated with rIFN- γ and DEX did not differ significantly from cultures treated with rIFN- γ alone (Figure 11, bottom panel: Treatments 5 and 6 were not found to be significantly different from Treatments 2 and 3, respectively). The data shown in Figure 12 were derived from 3 experiments in which macrophages were cultured in the presence of rIFN- γ and/or DEX (5 x 10⁻⁶M or 1 x 10⁻⁵M). Steady-state levels of A_{α} -specific mRNA were assayed after 24 hr. At concentrations of rIFN- γ that alone induced suboptimal (0.5 U/ml) and optimal (5.0 U/ml) levels of A_{α} -specific mRNA, simultaneous treatment with 1 x 10⁻⁵M DEX led to a significant reduction in steady-state levels of A_{α} -specific mRNA. Northern blot analysis (Figure 13) failed to demonstrate smaller A_{α} -specific RNA species which might suggest degradation of the A_{α} -specific mRNA in cultures treated with DEX.

Down-regulation of rIFN- γ -induced levels of other I-region mRNA by IFN- α/β and DEX. Given the finding that treatment with rIFN- γ led to increased steady-state levels of A_{α} -, A_{β} -, and E_{α} -specific mRNA (Table 4), antagonism of rIFN- γ -induced Ia expression by IFN- α/β and DEX was also examined for these other class II MHC loci. Cytoplasmic RNA was isolated from macrophage cultures that had been treated with 5.0 U/ml rIFN- γ in the absence or presence of 100 U/ml IFN- α/β (Table 6) or 1 x 10⁻⁵M DEX (Table 7). In both experiments, the RNA was applied directly to nitrocellulose and analyzed using A_{α} , A_{β} , and E_{α} , and DHFR cDNA probes. The data from Tables 6 and 7 illustrate that treatment with IFN- α/β and DEX, respectively, led to the antagonism of rIFN- γ -induced, steady-state levels of A_{α} -, A_{β} -, and E_{α} -specific mRNA. Due to the variability in labeling efficiency of the different I-region cDNA probes inherent in this type

Figure 11. Effect of rIFN- γ , DEX, and rIFN- γ plus DEX treatment on the steady-state levels of A_{α} - and DHFR-specific mRNA. Cytoplasmic or total RNA was isolated from macrophages treated for 24 hr with medium (Treatment 1), 0.5 U/ml rIFN- γ (Treatment 2), 5.0 U/ml rIFN- γ (Treatment 3), 1 x 10-5M DEX (Treatment 4), 0.5 U/ml rIFN- γ plus 1 x 10-5M DEX (Treatment 5), or 5.0 U/ml rIFN- γ plus 1 x 10-5M DEX (Treatment 6). The RNA preparations were either electrophoresed and transferred to nitrocellulose or applied directly to nitrocellulose and analyzed using A_{α} and DHFR cDNA probes. The number in parentheses above each treatment represents the number of individual experiments ("n") from which the average value was derived. The results represent the arithmetic means \pm S.D. of "n" experiments. The results were compared using an unpaired Student's *t*-test. With respect to relative Ia area (top graph), Treatments 5 and 6 differed significantly (p < 0.05) from Treatments 2 and 3, respectively. With respect to relative DHFR area (bottom graph), Treatments 5 and 6 did not differ significantly from Treatments 2 and 3, respectively.



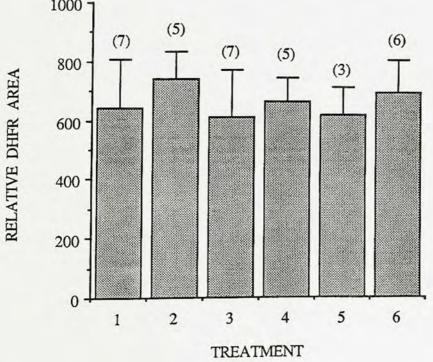
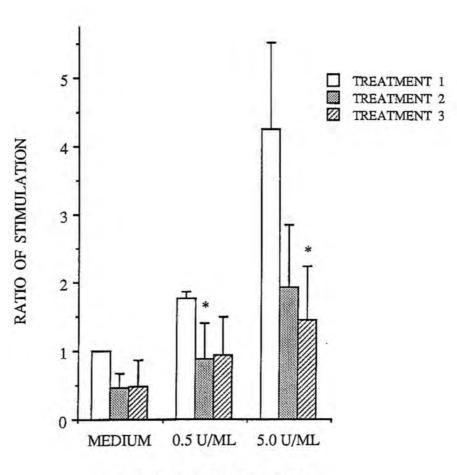
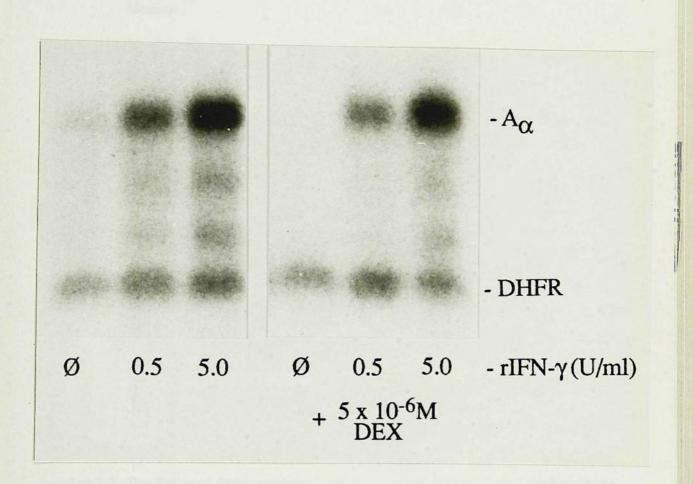


Figure 12. Effect of DEX on the steady-state levels of rIFN- γ -induced A_{α} -specific mRNA. Cytoplasmic RNA was harvested from macrophage cultures treated for 24 hr with medium or rIFN- γ (0.5 or 5.0 U/ml) in the absence of DEX (Treatment 1), in the presence of 5 x 10⁻⁶M DEX (Treatment 2), or in the presence of 1 x10⁻⁵M DEX (Treatment 3). The data were derived from both Northern and slot blots that had been analyzed using A_{α} and DHFR cDNA probes as described in Materials and Methods. The results represent the arithmetic mean ± S.D. of 3 separate experiments. The results were compared using an unpaired Student's *t*-test. An asterisk indicates that cultures treated with rIFN- γ (either 0.5 or 5.0 U/ml) and 1 x 10⁻⁵M DEX differed significantly (p < 0.05) from cultures treated with rIFN- γ only (either 0.5 or 5.0 U/ml).



rIFN- γ CONCENTRATION

Figure 13. Autoradiogram of Northern blot which illustrates the effect of DEX on the steady-state levels of rIFN- γ -induced A_{α} -specific mRNA. Total RNA was harvested from macrophage cultures that had been treated for 24 hr with medium, rIFN- γ (0.5 or 5.0 U/ml), DEX (5 x 10⁻⁶M), or combinations of rIFN- γ and DEX. The RNAs were then analyzed for A_{α} - and DHFR-specific mRNA as described in Materials and Methods.



	RATIO	RATIO OF STIMULATION		
	A_{α}	Aβ	E	
Medium	1.0 ^b	1.0	1.0	
5.0 U/ml rIFN-γ	4,2	10.4	5.3	
5.0 U/ml rIFN-γ+ 100 U/ml IFN-α/β	1.5	4.4	2.1	

^aCytoplasmic RNA was extracted from macrophages cultured for 24 hr in the presence of medium, 5.0 U/ml rIFN- γ , or 5.0 U/ml rIFN- γ plus 100 U/ml IFN- α/β . The RNA preparations were applied to slot blots and hybridized to the indicated I-region cDNAs. They were subsequently analyzed by procedures described in Materials and Methods.

^bThe Ia_{control}/DHFR_{control} value in the denominator of the Ratio of Stimulation formula was the medium-treated 24 hr value using the indicated probe (either A_{α} , A_{β} , or E_{α}). The Ratio of Stimulation values for medium-treated cultures analyzed with A_{α} , A_{β} , and E_{α} are all set equal to 1.0. The Ratios of Stimulation were derived from the arithmetic means of duplicate tracings from a single experiment which was representative of 2 separate experiments. Duplicate tracings were all within 5% of each other.

	RATIO	OF STIMUI	LATION
	A_{α}	A_{β}	Eα
Medium	1.0 ^b	1.0	1.0
5.0 U/ml rIFN-γ	4.6	6.5	8.7
5.0 U/ml rIFN-γ + 1 x 10 ⁻⁵ M DEX	2.9	4.9	6.2

^aCytoplasmic RNA was extracted from macrophages cultured for 24 hr in the presence of medium, 5.0 U/ml rIFN-γ, or 5.0 U/ml rIFN-γ plus 1 x 10⁻⁵M DEX. The RNA preparations were applied to slot blots and hybridized to the indicated I-region cDNAs. They were subsequently analyzed by procedures described in Materials and Methods.

bThe $Ia_{control}$ /DHFR_{control} value in the denominator of the Ratio of Stimulation formula was the medium-treated 24 hr value using the indicated probe (either A_{α} , A_{β} , or E_{α}). The Ratio of Stimulation values for medium-treated cultures analyzed with A_{α} , A_{β} , and E_{α} are all set equal to 1.0. The Ratios of Stimulation were derived from the arithmetic means of duplicate tracings from a single experiment which was representative of 2 separate experiments. Duplicate tracings were all within 5% of each other.

of analysis, it was not possible to draw any conclusions about the relative expression of one I-region gene compared to another I-region gene.

Demonstration of induction of A_{\gamma}-specific mRNA by rIFN-\gamma and its down-regulation by IFN-α/β and DEX in C3H/OuJ macrophages. C3H/HeJ mice possess a single gene defect, Lpsd, which renders them hyporesponsive to the effects of Gram negative endotoxins (reviewed in Morrison and Ryan, 1979; Vogel et al., 1981; Rosenstreich, 1985). Although all of the previously reported protein work had been carried out in LPS-responsive macrophages, as well as in C3H/HeJ macrophages, it was necessary to insure that the modulation of steady-state levels of A_{α} -specfic mRNA by rIFN- γ and the inhibitors, IFN- α/β and DEX, was not unique to C3H/HeJ macrophages. Macrophages derived from mice which were LPS-responsive (Lpsn), but syngeneic with C3H/HeJ mice, were also examined. For these studies, C3H/OuJ macrophages were thioglycollate-elicited, cultured, and treated identically as described previously for C3H/HeJ macrophages. Twenty-four hr following treatment with medium, rIFN-γ (0.5 or 5.0 U/ml), IFN- α/β (100 U/ml), DEX (1 x 10⁻⁵M) or combinations of rIFN- γ plus IFN- α/β or DEX, cytoplasmic RNA was isolated and analyzed using A_{α} and DHFR cDNA probes. Table 8 illustrates that like C3H/HeJ macrophages, there was a dose-dependent induction of steady-state levels of Aa-specific mRNA in response to rIFN-y. In addition, the data demonstrate that simultaneous treatment of C3H/OuJ macrophages with rIFN- γ and IFN- α/β or DEX resulted in markedly reduced levels of steady-state A_{α} -specific mRNA when compared to the levels of steady-state A_{α} -specific mRNA induced in the presence of rIFN-γ alone.

Effect of IFN- α/β and DEX on rIFN- γ -induced cytoplasmic and nuclear levels of A_{α} -specific RNA. To examine the nature of the antagonism of IFN- α/β and DEX on rIFN- γ -induced A_{α} -specific mRNA levels, nuclear and cytoplasmic RNA species were

TABLE 8 $\frac{\text{Induction of A}_{\alpha}\text{-Specific mRNA by rIFN-}\gamma \text{ and Its Down-Regulation by }}{\text{IFN-}\alpha/\beta \text{ and DEX in C3H/OuJ Macrophages}^a}$

Dose of rIFN-γ (U/ml)	Inhibitor	Ratio of Stimulation	
0	0	1.0b	
	IFN- α/β (100 U/ml)	0.7	
	DEX (1 x 10 ⁻⁵ M)	1.0	
0.5	0	4.1	
	IFN-α/β (100 U/ml)	1.1	
	DEX (1 x 10 ⁻⁵ M)	1.2	
5.0	0	6.7	
	IFN-α/β (100 U/ml)	3.5	
	DEX (1 x 10 ⁻⁵ M)	3.7	

aCytoplasmic RNA was extracted from C3H/OuJ macrophages cultured for 24 hrs in the presence of medium, rIFN- γ (0.5 or 5.0 U/ml), or rIFN- γ (0.5 or 5.0 U/ml) in combination with IFN- α /β (100 U/ml) or DEX (1 x 10⁻⁵M).

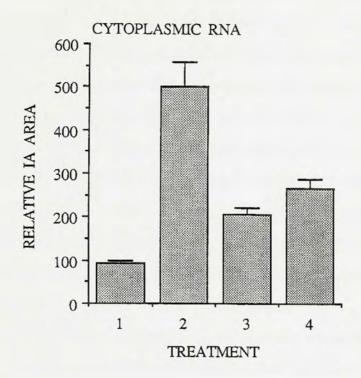
bThe Ia_{control}/DHFR_{control} value in the denominator of the Ratio of Stimulation formula was the medium-treated 24 hr value. Thus, the Ratio of Stimulation for medium-treated cultures is equal to 1.0. The results are expressed as the arithmetic mean of a single experiment representative of 2 separate experiments. Duplicate tracings were always within 5% of each other.

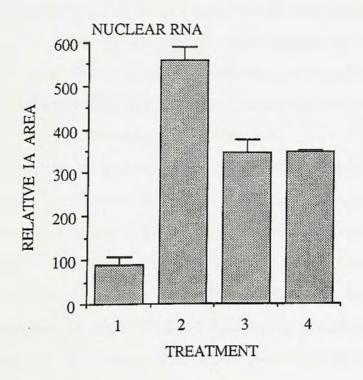
analyzed. To determine whether the modulation of rIFN-y-induced cytoplasmic levels of A_{α} -specific mRNA by IFN- α/β and DEX was also reflected in nuclear RNA populations, cytoplasmic and nuclear RNA from cultures treated with rIFN-γ alone and in combination with either IFN-α/β or DEX was prepared and analyzed. The data presented in Figure 14 were derived from the densitometric tracings of slot blots of RNA samples (both cytoplasmic and nuclear) hybridized with an A_{α} -specific cDNA probe. Since DHFR signal intensities were extremely low in the nuclear preparations, the Ratio of Stimulation was calculated using only the relative areas which correspond to the digitized tracings of A_{α} -specific signals. In addition, to achieve comparable A_{α} -specific signals between cytoplasmic and nuclear preparations, approximately ten times more nuclear RNA (30 µg per slot) than cytoplasmic RNA (3 µg per slot) was applied to the nitrocellulose. From the data, it is clear that the alterations in levels of rIFN- γ -induced A_{α} -specific mRNA in the presence of the inhibitors IFN- α/β and DEX are also reflected in the nuclear RNA species. The magnitude of the antagonism is comparable in the cytoplasm and nuclear compartments as well. Thus, it appears that there is no dramatic alteration in the rates of transport of A_{α} -specific mRNA from the nucleus into the cytoplasm in the presence of either inhibitor. Taken collectively, these observations suggested a transcriptional role of the inhibitors in the modulation of A_{α} -specific gene expression.

NUCLEAR TRANSCRIPTION ANALYSIS OF I REGION-SPECIFIC RNA

Given the finding that the alterations in steady-state levels of Ia-specific RNA induced by rIFN- γ or rIFN- γ plus IFN- α/β or DEX were observed both in the cytoplasm and the nucleus (Figure 14), transcription rate studies were performed to determine if the inhibitors could modulate directly the rate of I-region gene transcription.

Figure 14. Comparison of the cytoplasmic and nuclear steady-state species of rIFN- γ -induced A_{α} -specific RNA in the presence of IFN- α/β or DEX. Cytoplasmic and nuclear RNA species were isolated from macrophages treated with medium only (Treatment 1), 5.0 U/ml rIFN- γ (Treatment 2), 5.0 U/ml rIFN- γ plus 100 U/ml IFN- α/β (Treatment 3), and 5.0 U/ml rIFN- γ plus 1 x 10-5M DEX (Treatment 4). For cytoplasmic RNA species, 3 μ g/slot was applied and for nuclear RNA species, 30 μ g/slot was applied. Both blots were hybridized with the A_{α} cDNA probe. The relative Ia area values represent the arithmetic means \pm S.D. of duplicate slots per treatment for a single experiment. The results are representative of 3 separate experiments.





Establishment of the parameters to examine transcription rates in isolated nuclei. In general terms, the measurement of the rate of transcription of a particular gene involves three distinct procedures. They are: (i) isolation and purification of nuclei (using sucrose fractionation methods followed by non-ionic solubilization of the nuclei), such that the nuclear envelope is permeabilized sufficiently to allow for the diffusion of triphosphates and cations while the metabolic integrity of the preparation is maintained; (ii) in vitro elongation of the nuclear transcripts already initiated in situ in the presence of radiolabeled RNA precursors (³²P-UTP), followed by purification of the labeled transcripts away from contaminating proteins and DNA in the nuclei; and, (iii) detection of specifically-labeled transcripts among the entire population of elongated transcripts with hybridization methods which involve immobilization of specific cDNA sequences onto nitrocellulose filters followed by densitometric quantitation of the autoradiograms.

To study rates of gene transcription, a number of different parameters first had to be optimized to insure that the data obtained would be interpretable and not artifactual. These included: (i) demonstration of linearity of transcript binding to DNA's immobilized onto filters; (ii) determination of optimum length of hybridization period of transcripts with cDNAs immobilized on filters; (iii) determination of hybridization efficiency of transcript binding to DNA's immobilized on filters; (iv) demonstration of significant levels of RNA polymerase II activity in nuclei preparations; and, (v) demonstration of the strand specificity of the transcripts synthesized in isolated nuclei.

In order to detect the specific, radiolabeled transcripts synthesized during the $\underline{\text{in}}$ $\underline{\text{vitro}}$ transcription elongation assay, cDNAs were immobilized onto nitrocellulose filters and hybridizations were performed. The immobilized cDNAs were hybridized with the $^{32}\text{P-}$ or $^{3}\text{H-labeled}$, complementary RNA sequences and the hybridization products were then analyzed by liquid scintillation counting or by autoradiography. The first three parameters were optimized using a $^{3}\text{H-labeled}$, A_{α} -transcript (homologous to mRNA) that was synthesized in a T7 polymerase-directed $\underline{\text{in}}$ $\underline{\text{vitro}}$ transcription reaction (for details of its

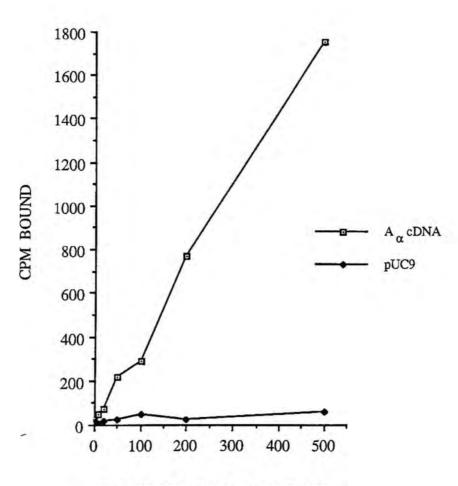
synthesis see Materials and Methods). The last two parameters were examined in an <u>in</u> <u>vitro</u> transcription elongation assay using nuclei isolated from C3H/HeJ macrophages (as described in Materials and Methods).

To insure that the cDNAs immobilized onto the nitrocellulose filters were in excess during the hybridization reactions, increasing quantities of radiolabeled (3 H) A_{α} -transcripts ("message-sense") were hybridized for 24 hr to filters which contained 1 μ g per slot of an A_{α} cDNA plasmid or 1 μ g of an irrelevant plasmid (pUC9). Figure 15 demonstrates that the binding of radiolabeled A_{α} -transcript is linear over a wide range of input RNA (1 - 500 ng). Linearity over this range is adequate to insure cDNA excess on the nitrocellulose filters since it is highly unlikely that quantities of RNA greater than 100 ng would be generated in an in vitro transcription assay (Dr. Daniel Schoenberg, personal communication). Radiolabeled A_{α} -transcripts did not bind significantly to any pUC9 sequences which demonstrated the sequence specificty of the binding (i.e., the greatest A_{α} -signal binding to pUC9 sequences was 59 cpm and was seen only on filters to which 500 ng of labeled RNA was hybridized).

To determine the optimal length of the hybridization period, the hybridization of radiolabeled A_{α} -transcripts ("message-sense") to filters which contained A_{α} cDNA and irrelevant sequences (identical to those used in the cDNA excess study) was monitored over a 4 day period. Figure 16 illustrates that maximum hybridization of A_{α} -transcripts to immobilized A_{α} cDNA sequences occurs after 2 to 3 days incubation at 40°C. In addition, no increase in transcript hybridization to the irrelevant plasmid, pUC9, was noted with increasing hybridization time. Since binding remained constant between 2 and 3 days of hybridization with no significant increase in nonspecific binding, all subsequent hybridizations were performed for 2 to 3 days.

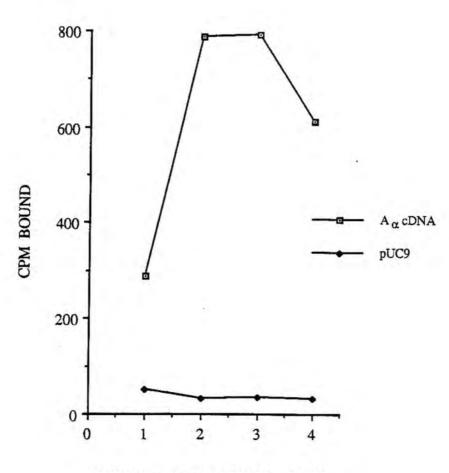
The efficiency of transcript hybridization to immobilized cDNAs on nitrocellulose was examined in the following manner. One-hundred ng of radiolabeled A_{α} -transcript ("message-sense") was applied directly to nitrocellulose and was immobilized. The

Figure 15. Demonstration of the linearity and specificity of A_{α} -transcript binding to an immobilized plasmid which contains an A_{α} cDNA insert. Increasing amounts (1 - 500 ng) of ${}^3\text{H-A}_{\alpha}$ -transcripts (generated from an in vitro T7-directed polymerization reaction) were hybridized to nitrocellulose filters which contained 1 μ g/slot of a plasmid with an A_{α} cDNA insert (pCEXV- A_{α}) or an irrelevant plasmid, pUC9. The filters were then washed, air-dried, and counted in non-aqueous scintillation fluid. The results represent the cpm bound to each filter and are representative of 2 separate experiments.



RNA APPLIED TO FILTERS (NG)

Figure 16. Determination of the optimal hybridization period of A_{α} -transcript binding to an immobilized plasmid which contains an A_{α} cDNA insert. One hundred ng of ${}^3\text{H-A}_{\alpha}$ transcript were hybridized for varyings periods of time to filters which contained 1 mg/slot of plasmid containing an A_{α} cDNA insert or an irrelevant plasmid, pUC9. At the indicated times, the filters were washed, air-dried, and counted in non-aqueous scintillation fluid. The results represent the cpm which bound to each filter and are representative of 2 separate experiments.



HYBRIDIZATION PERIOD (DAYS)

radioactivity associated with 100 ng of radiolabeled transcript bound directly to nitocellulose was 5521 cpm, as determined by liquid scintillation counting. This value was compared to the cpm associated with filters (which contained the immobilized, unlabeled A_{α} cDNA sequence) that had been hybridized for 2 days with radiolabeled A_{α} -transcript (100 ng). The cpm associated with these filters was 789. Thus, the efficiency of hybridization was calculated as ~ 14% (789/5521). This efficiency is consistent with previously published studies which utilized similar hybridization systems (Martin et al., 1986).

In nonproliferating cells, RNA polymerase II is responsible for a significant proportion of total polymerase activity (Darnell et al., 1986). As an additional control to demonstrate the fidelity of the in vitro transcription system, relative RNA polymerase II activity was determined by comparing TCA-precipitable counts of transcripts that were elongated (see Materials and Methods) in the absence or presence of $0.5 \mu g/ml$ α -amanitin. This concentration of α -amanitin has been shown previously to inhibit RNA polymerase II activity without affecting RNA polymerase I or III activity (Kedinger et al., 1970; Roeder, 1974; Darnell et al., 1986). The data shown in Table 9 demonstrate that there is an $\sim 50\%$ reduction in the cpm associated with total nuclear transcripts in the presence of $0.5 \mu g/ml$ α -amanitin. This verifies that a significant fraction of the polymerase activity in these nuclei is the result of RNA polymerase II transcription and is consistent with previous reports of the contribution of RNA polymerase II activity to total polymerase activity in nonproliferating cells (Darnell et al., 1986).

To insure that the transcripts that were synthesized in the <u>in vitro</u> elongation assay were transcribed from the "sense" strand of DNA (i.e., transcripts were of "message-sense"), the following strand specificity experiment was performed. Transcripts that had been synthesized in nuclei from medium- and rIFN- γ -treated macrophage cultures were hybridized to filters which contained single-stranded E $_{\beta}$ cDNA sequence of either "message-sense" (E $_{\beta}$ "+") or "anti-sense" (E $_{\beta}$ "-"). The rationale for using these

TABLE 9

Inhibition of RNA Polymerase II Activity In Nuclei Preparations by α-Amanitin^a

	³ H-UTP Incorporation ^a (cpm/culture)			
Treatment	Experiment 1	Experiment 2		
NONE	1170 ± 16 ^b	1130 ± 80		
α-Amanitin (0.5 μg/ml)	621 ± 104	579 <u>+</u> 89		
duction in RNA polymerase II activity	47%	49%		

 $^{^{}a}$ A $_{\alpha}$ transcripts were elongated in the presence of 3 H-UTP as described in the Materials and Methods in the absence (NONE) or presence of α -amanitin. Incorporation of 3 H-UTP into total nuclear transcripts was compared following TCA precipitation using non-aqueous liquid scintillation counting.

^bResults represent the arithmetic mean \pm S.D. of duplicate cultures.

single-stranded sequences was as follows: Ia-specific RNA is typically transcribed from only one strand of duplex DNA. However, in an $\underline{\text{in vitro}}$ RNA elongation system, such as that utilized in these experiments, transcription may generate species which are not produced naturally. E_{β} transcripts generated in the elongation assay that are of the correct polarity should bind only to complementary sequences or "anti-sense" cDNAs (E_{β} "-"). Binding of these transcripts to "message-sense" cDNAs (E_{β} "+") would indicate artifactual transcription (i.e., the transcript was being synthesized from the DNA strand not normally used $\underline{\text{in vivo}}$). Figure 17 illustrates the autoradiographic results of hybridizations of transcripts (isolated from nuclei derived from medium- or rIFN- γ -treated macrophage cultures) to either "message-sense" ("+") or "anti-sense" ("-") E_{β} sequences. In nuclei isolated from rIFN- γ -treated cultures, there is a significant preference of binding of radiolabeled transcripts to the E_{β} "-" sequence when compared with binding to the E_{β} "+" sequence. This indicates that E_{β} -transcripts that are elongated in this defined system are the correct polarity.

Effect of rIFN- γ treatment on the rates of I-region gene transcription. The previous experiment in which it was demonstrated that rIFN- γ treatment led to an accumulation of A_{α} -specific message in both the nucleus and the cytoplasm (Figure 14), suggested that a transcriptional mechanism might be involved in the induction of A_{α} -specific mRNA by rIFN- γ . To determine if rIFN- γ modulated I-region gene transcription, the following nuclear "run-on" experiment was performed. Nuclei were harvested from macrophage cultures that were treated with medium or rIFN- γ (5.0 U/ml) for varying periods of time (i.e., 4, 12, 24, or 48 hr). Once all the nuclei were prepared, they were incubated (for 45 min at 32°C) in a reaction mixture which contained 32 P-UTP and other components shown previously to be necessary for the in vitro synthesis of RNA (i.e., unlabeled triphosphates, CTP, GTP, and ATP as precursors for RNA synthesis; the proper salt and buffer conditions of KCl, MgCl₂, MnCl₂, and Tris for optimal RNA

Figure 17. Autoradiogram from a nuclear "run-on" assay which illustrates the strand-specificity of binding to immobilized E_{β} "+" ("message-sense") of E_{β} "-" ("anti-sense") single-stranded DNA's of E_{β} transcripts elongated in vitro . ^{32}P -RNA transcripts were isolated following the elongation of macrophage nuclei that had been treated for 24 hr with medium only or rIFN- γ (5.0 U/ml). These transcripts were subsequently isolated and hybridized to filters which contained single-stranded DNA's derived from M13mp9 E_{β} "+" or from M13mp9 E_{β} "-" constructs. The filters were washed, air-dried, and exposed to film for autoradiography. The relative area value which corresponded to the digitized tracings from medium-treated cultures was 125 for M13mp9 E_{β} "+" and 157 for M13mp9 E_{β} "-". The relative area value which corresponded to the digitized tracings from rIFN- γ -treated cultures was 212 for M13mp9 E_{β} "+" and 471 for M13mp9 E_{β} "-". The E_{β} probes were used rather than the A_{α} probes because the E_{β} M13 constructs were available.

Treatment

MEDIUM

rIFN-γ

$$M13mp9E_{\beta}{}^{d}$$
"+" - $M13mp9E_{\beta}{}^{d}$ "-" -

polymerase II activity; and, the polyamines, spermine and spermidine, to minimize degradation due to endogenous RNases). The radiolabeled transcripts generated in the <u>in vitro</u> reaction were subsequently isolated from other nuclear components (as detailed in Materials and Methods). The purified ³²P-labeled transcripts were denatured and added to nitrocellulose filters to which the plasmids with I-region cDNA inserts, a DHFR cDNA insert, or an irrelevant plasmid had been applied in excess. The filters were then washed and the specific autoradiographic signals were quantified densitometrically.

The major assumptions associated with nuclear "run-on" assays are: (i) the amount of radiolabeled transcripts generated in a "run-on" assay reflects the number of gene transcripts pre-initiated at a particular time, and (ii) that the relative level of pre-initiated transcripts reflects the degree of RNA polymerase loading of a gene at a particular time (i.e., the rate of transcription of a gene). Given these assumptions, it is legitimate to relate the amount of radiolabeled transcripts generated from nuclei (isolated at a particular time) to the rate of transcription of that specific gene (at that time). Figure 18 shows the autoradiogram from a representative nuclear "run-on" experiment. Examination of the levels of elongated transcripts from nuclei derived from medium-treated macrophages (at 4 and 48 hr) revealed a relatively low basal rate of I-region gene transcription in these cultures. The levels of elongated transcripts observed in nuclei derived from macrophages cultured in the presence of rIFN-y for 12 hr, demonstrated an intermediate rate of transcription. Maximal induction of I-region gene transcription occurred 24 hr following treatment with rIFN-y. The levels of elongated transcripts observed in nuclei derived from macrophages cultured in the presence of rIFN-y for 48 hr were reduced in comparison to the 24 hr levels. This suggests that the rate of I-region transcription declines between 24 and 48 hr. The decline in the rate of I-region gene transcription between 24 and 48 hr, together with the demonstration of elevated levels of steady-state Ia-specific mRNA between 24 and 48 hr (Figure 3 and Table 2) suggest that Ia-specific mRNA's have a relatively long half-life (i.e., > 24 hr).

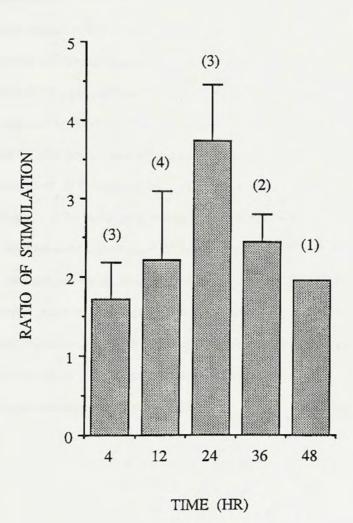
Figure 18. Autoradiogram from a nuclear "run-on" assay which illustrates the effect of rIFN- γ on the rate of I-region gene transcription. At the indicated times, nuclei were harvested from macrophages treated with medium only or rIFN- γ (5.0 U/ml). These nuclei were elongated in the presence of ³²P-UTP, and the resulting transcripts were harvested. The transcripts were then hybridized for 2 days to nitrocellulose filters which contained plasmids with A_{α} , A_{β} , E_{α} , and E_{β} cDNA inserts. The filters were washed, air-dried, and exposed to film for autoradiography. The results are representative of several nuclear "run-on" experiments which are pooled in Figure 19.

Treatment	MEDIUM		rIFN-γ			
hr	4	48	4	12	24	48
I-region -	MACRICAL	C	or Magazine	Montelying		arcana ;
DHFR -	-	**********	*CONTRACTORS*	-	-	Recorded .
pSP64 -	(Address)		Marine L	1	demonstrative and	(

Given the relative constancy of the transcription rate of DHFR in this system (Figure 18), the relative levels of bound DHFR transcripts provided an internal control analogous to that used in the steady-state RNA studies. The Ratio of Stimulation (see Materials and Methods) again provided a useful formula by which the rates of transcription of different samples could be related after controlling for variations in signal intensity that may have been caused by differences in recovery of transcripts between sample treatments. In addition, the irrelevant plasmid sequence, pSP64, provided a negative control to monitor the nonspecific binding activity among transcript samples that may have resulted from unequal handling of transcripts during their isolation and purification (i.e., variations in washing of the filters after TCA-precipitation or differences in DNA and protein contamination following enzymatic digestion and organic extraction of the samples). An analysis of pooled data from 4 separate "run-on" experiments in which the effect of rIFN-y on the rate of I-region gene transcription was examined is illustrated in Figure 19. The data demonstrate that 24 hr following rIFN-y treatment there was a 3.7-fold increase in the rate of I-region gene transcription. The data are consistent with the rate kinetics described earlier (Figure 3 and Table 2).

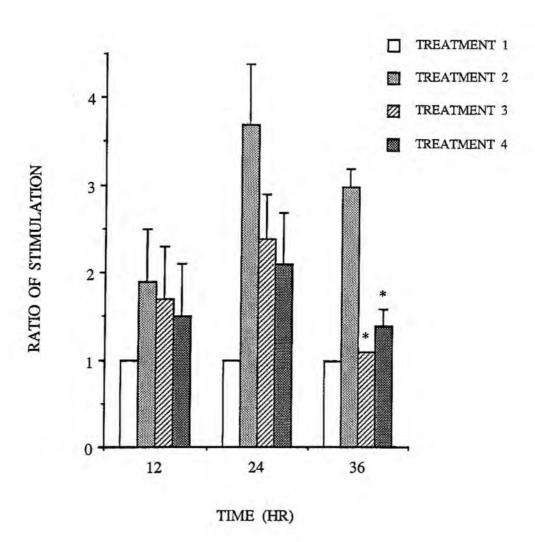
Effect of IFN- α/β and DEX on rIFN- γ -induced rates of I-region gene transcription. The steady-state analysis of nuclear RNA samples from rIFN- γ plus inhibitor-treated cultures (both IFN- α/β and DEX) suggested that the inhibitors acted via a transcriptional mechanism to down-regulate rIFN- γ -induction of A_{α} -specific expression. To investigate whether the reduction in rIFN- γ -induced steady-state levels of Ia-specific mRNA seen with IFN- α/β and DEX treatment were caused by reductions in the rate of I-region gene transcription, the rate of transcription in nuclei derived from cultures treated with rIFN- γ , rIFN- γ plus IFN- α/β , or rIFN- γ plus DEX treated cultures were compared. Nuclei were harvested from macrophage cultures treated with medium, rIFN- γ (5.0 U/ml), IFN- α/β (100 U/ml), DEX (1 x 10⁻⁵M) or rIFN- γ plus IFN- α/β or DEX for varying

Figure 19. Effect of rIFN- γ on the rate of I-region gene transcription. As described in Figure 18, nuclei were harvested from macrophages (previously treated with medium only or 5.0 U/ml rIFN- γ for the indicated periods of time). The transcripts were then elongated in vitro and harvested. I-region-specific transcripts were selected by hybridization of total transcripts to filters which contained plasmids with I-region inserts. After hybridization, washing, and drying, the filters were exposed to X-ray film. The autoradiographic signals were quantified and pooled. The number in parentheses above each treatment represents the number of individual experiments ("n") from which the average value was derived. The results represent the artihmetic means \pm S.D. The results were compared using an unpaired Student's *t*-test. Only the 24 hr value was found to be significantly different (p < 0.05) from the 4 hr value.



periods of time (i.e. 12, 24, and 48 hr). Figure 20 illustrates the pooled Ratio of Stimulation data from 2 independent "run-on" experiments in which the effects of DEX and IFN- α/β on the rate of I-region gene transcription induced by rIFN- γ were examined. Since the amount of DHFR-specific transcripts generated in the in vitro elongation assay did not vary following treatment of rIFN-γ plus DEX or rIFN-γ plus IFN-α/β (data not shown), the amounts of DHFR-specific transcripts generated again provided an internal control to equalize for variations in signal intensities that may have been caused by transcript recovery differences between samples. In the presence of rIFN-y plus DEX (Treatment 3) or rIFN- γ plus IFN- α/β (Treatment 4), the measurable increase in the rate of transcription induced by rIFN-y alone (Treatment 2) over basal rate (medium only, Treatment 1) in a 12 hr period was relatively unaltered. However, after 24 hr of treatment with a combination of rIFN-γ plus IFN-α/β or rIFN-γ plus DEX, there was an inhibition (i.e., an ~ 40% and ~ 35% reduction, respectively) of the rate of I-region gene transcription. The level of significance of the inhibition with IFN- α/β and DEX after 24 hr was p = 0.06, as assessed by an unpaired Student's t-test. The inhibition by DEX and IFN- α/β was even more striking after 36 hr of culture (i.e., > 50% reduction and p values < 0.05 with both inhibitors). These data support the hypothesis that the induction of I-region gene products by rIFN-y, as well as its modulation by the inhibitors DEX and IFN-α/β, are regulated transcriptionally.

Figure 20. Effect of IFN- α/β and DEX on rIFN- γ -induced rates of I-region gene transcription. Nuclear "run-on" experiments were performed on nuclei isolated from macrophages that had been treated for the indicated periods of time with medium only (Treatment 1), 5.0 U/ml rIFN- γ (Treatment 2), 5.0 U/ml rIFN- γ plus 100 U/ml IFN- α/β (Treatment 3), or 5.0 U/ml rIFN- γ plus 1 x 10-5M DEX (Treatment 4). The transcripts from the nuclei were elongated in vitro, harvested, and hybridized to filters which contained plasmids with I-region inserts, a DHFR insert, and an irrelevant sequence. After hybridization, washing, and drying, the filters were exposed to X-ray film. The autoradiographic signals were quantified and a Ratio of Stimulation was determined. The results represent the arithmetic means \pm S.D. of 2 separate experiments. The results were compared using an unpaired Students's *t*-test and an asterisk above a value indicates that it differed significantly (p < 0.05) from the Treatment 1 value at the same time point.



DISCUSSION

At the onset of this study, little was known about the molecular mechanisms which underlie the induction and down-regulation of class II MHC antigens (Ia antigens in the mouse). However, a number of studies have been published recently which have begun to provide insights into the nature of the regulation of class II MHC antigen expression. In this discussion, I will first present a synopsis of recent data which address molecular mechanisms which may contribute to the induction and/or down-regulation of Ia antigen expression. The work presented in this dissertation will then be discussed in the context of these studies.

INDUCTION OF IA ANTIGEN EXPRESSION

Ia antigens are transmembrane glycoproteins which are limited typically to the surface of antigen-presenting cells of the immune system, such as macrophages, dendritic cells, B cells, and certain T cell subpopulations. The ability of macrophages and other cells to present antigen appropriately has been correlated with their ability to express these antigens on their cell surface. Conversely, a paucity of Ia antigens has been shown to preclude appropriate presentation function (reviewed in Unanue, 1984; Unanue and Allen, 1987). Early work (Birmingham et al. 1982; Beller and Unanue, 1981; Beller and Ho, 1982) demonstrated that the expression of Ia antigen was not a constitutive phenomenon on most cell types, but rather, was inducible and transient. It is now recognized that the induction of Ia antigen, as well as maintenance of its expression, is primarily lymphokine-mediated (Steinman et al., 1980; Steeg et al., 1980), and that the principal agent in lymphokine supernatants that is responsible for the induction of Ia antigen expression on

macrophages is IFN-γ (Steeg et al., 1982a; King and Jones, 1983; Wong et al., 1984; Kelley et al., 1984; Warren and Vogel, 1985a). However, other substances have since been shown to induce Ia antigen expression (i.e., GM-CSF, IL 4, MDP, and certain viruses) or to synergize with IFN-γ to induce Ia antigen expression (i.e., TNF and 1,25(OH)₂D₃). A common pathway for Ia antigen induction by IFN-γ, TNF, MDP, GM-CSF, certain viruses, and IL 4 has not yet been elucidated. This spectrum of agents may represent a class of substances that induces the expression of a common intermediate which, in turn, is responsible for the induction of Ia antigen expression. Alternatively, these substances may initiate several distinct pathways for induction of Ia antigen expression.

Data derived from virus induction studies suggest that distinct pathways for the induction of Ia antigen may exist. For example, the JHM strain of coronavirus appears to induce Ia antigen expression on astrocytes via a mechanism that is independent of viral replication or the production of virus-induced interferon (Massa et al., 1986). In contrast, Kennedy et al. (1985) have shown that lentivirus-infected peripheral blood mononuclear cultures produce a species of interferon (LV-IFN) which results in the induction of Ia antigen expression on uninfected macrophages. Using a similar system with a different strain of visna virus, Narayan et al. (1985) demonstrated that the Ia-inducing interferon produced following viral infection shares properties with both type I and II interferons: LV-IFN resembles type I interferons (i.e., IFN-α and IFN-β) in its stability at heat and low pH, molecular size, and nonglycosylated nature. However, the requirement for the presence of macrophages and T cells for the production of LV-IFN, as well as its ability to induce Ia antigen, is more reminiscent of IFN-γ (reviewed in Friedman and Vogel, 1983).

Crawford et al. (1987) have recently shown that peritoneal macrophages respond to the lymphokine IL 4 to express Ia antigens. It is not yet clear whether IL 4 and IFN-γ, which are both T cell-derived lymphokines and which are both capable of inducing Ia antigen expression on macrophages, act to induce Ia antigen expression through a common

inductive pathway or by independent pathways. The kinetics and the levels of induction of Ia antigen were found to be similar for both lymphokines, but this does not prove a common inductive mechanism. Although 10.0 U/ml of IL 4 and 0.1 U/ml of IFN-y each resulted in 50% maximal Ia antigen expression, it was not possible to compare directly the relative efficacies of these two lymphokines given that the bioassays used for the determination of their respective activities were quite different. It is also interesting to note that IL 4 has been shown to be the principal inducer of Ia antigen expression in normal B cells and can induce Ia in many B cell lines (Noelle et al., 1986; Polla et al., 1986a, 1984b), while IFN-γ is not an effective inducer of Ia antigen on these cell types (Noelle et al., 1984; Roehm et al., 1984; Polla et al., 1986a). Polla et al. (1986a) demonstrated that IL 4 treatment of an Abelson virus-transformed B cell line, R8205, results in a very rapid (within 1 hr) induction of A_α- and I_i-specific mRNA; however, the addition of cycloheximide did not prevent the accumulation of Ii-specific mRNA (Ax-specific mRNA levels were not measured in the presence of cycloheximide). This is in contrast to the protein dependency phase demonstrated herein for the induction of A_{\alpha}-specific RNA on macrophages by IFN-γ (Figure 7).

Cell fusion studies between B cells or between B cells and macrophages have also helped to elucidate the molecular mechanisms by which Ia antigen is induced. Polla et al. (1986b) demonstrated that fusion of a constitutively DR-positive, human B cell lymphoma to an Ia-negative, pre-B cell, resulted in the expression of murine I-A and I-E antigens on the hybrids. Thus, the human B cell lymphoma appparently provided an appropriate trans-activating signal to the murine class II MHC genes. This same approach was taken to address the nature of the induction of class II MHC antigen expression in different cell types. Maffei et al. (1987) has illustrated that a somatic cell hybrid made between a human DR-negative B cell variant (RJ 2.2.5) and a murine Ia-negative macrophage-like cell line (P388D₁) responded to murine IFN-γ with an increase in murine Ia-specific mRNA and protein. However, there was no coordinate induction of human DR-specific mRNA

and protein. In contrast, Accolla et al. (1985a; 1985b) demonstrated the constitutive expression of human class II MHC-specific mRNA and protein in somatic cell hybrids between the same human DR-negative B cell variants used by Maffei et al. (1987) and either murine B lymphoma cells or murine spleen cells. Thus, in light of the findings of Maffei et al. (1987), Polla et al. (1986b), and Accolla et al. (1985a; 1985b), it is likely that the intracellular signals which regulate class II MHC antigen expression in macrophages and B cells are distinct and incapable of complementing one another.

Little is known about the role of intracellular second signals in the induction of Ia antigen expression by IFN-y. Signal transduction may occur via a mechanism similar to many peptide hormones in that the binding of IFN-γ to its receptor causes the production of second messengers which modulate gene activity through enzymatically-linked phosphorylation and/or dephosphorylation of intracellular proteins. Alternatively, the Ia genes or the gene(s) which code(s) for the trans-activating factor(s) may be activated by the direct interaction of internalized IFN-γ or an internalized IFN-γ-receptor complex (ligand-receptor complex) with the gene. The possible involvement of second messengers in the activation of macrophages by IFN-y has been examined by several investigators. Straussman et al. (1986) have shown that phorbol esters, such as phorbol myristate acetate (PMA), and/or the Ca⁺² ionophore, A23187, can mimic the inductive effects of IFN-y on Ia antigen expression on murine peritoneal macrophages. Thes findings suggests that protein kinase C (which is stimulated directly by PMA; Nishizuka, 1984) and/or alterations in intracellular Ca+2 levels may be involved in the intracellular signalling process which leads to the induction of Ia antigen expression. Since protein kinase C and Ca+2 levels are the targets of the second messengers diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP₃), respectively, it has been hypothesized that binding of IFN-γ to its receptor may stimulate the hydrolysis of phosphotidylinositol-4,5-bisphosphate (PIP2) which leads to the production of DAG and IP3 (Weiel et al., 1985). Presently, there is no direct evidence that IFN-y treatment leads to increased PIP2 hydrolysis in macrophages. However, Yap et

al. (1986) have provided evidence in support of a role for DAG and IP₃ in the signalling process by IFN- γ . They demonstrated that there is an immediate increase (within 30 sec) in DAG and IP₃ following treatment of fibroblasts with IFN- γ . Furthermore, they demonstrated that only cells which express receptors for IFN- γ respond with immediate increases, which suggests that the observed metabolite increases are dependent upon ligand-receptor interaction. Both IFN- α and IFN- β treatment of cells which express receptors for these IFNs, also resulted in very rapid increases in DAG and IP₃ (Yap et al., 1986); however, neither IFN- α nor IFN- β induce murine Ia antigen expression (Ling et al., 1985). Thus, it is feasible that the second messengers act in concert with other signals induced by either IFN- γ or IFN- α/β , which, in turn, modulate gene activity uniquely. Further experiments in which specific intracellular signal antagonists are shown to block IFN- γ -induced Ia antigen expression will be required to demonstrate the participation of second messengers in this inductive cascade.

In addition to the induction of class II MHC antigen on immune cell types, IFN- γ has been shown to induce class II MHC antigen expression on a number of non-immune cell types. For example, the ability of IFN- γ to induce class II MHC antigens on endocrine cells (i.e., thyroid epithelial cells; Botazzo et al., 1983 and pancreatic β -cells; Pujol-Borell et al., 1987) has led to some interesting models about the role of these populations in autoimmune states (i.e., Grave's thyrotoxicosis and type I diabetes mellitus, respectively). In these disorders, it is likely that the induction of class II MHC antigens on endocrine cells leads to an increased risk of presentation of self-antigens to auto-reactive T cells and to the initiation of an autoimmune cascade (Bottazzo et al., 1983). Similar models of self-reactivity have been proposed in the graft rejection process. For example, keratinocytes have been shown to express class II MHC antigens following a graft of incompatible lymphocytes. Following expression of Ia antigen, the keratinocytes are the target of the rejection process (Lampert et al., 1981).

Due to the potential role of class II MHC antigen expression on non-immune cells in states of autoimmunity and graft rejection, a great deal of recent investigation has focused on the mechanisms by which class II MHC antigen expression is down-regulated. Given that these self-destructive processes occur in a stimulus-rich, immunoreactive environment, it is particularly relevant to study the mechanisms involved in the down-regulation of class II MHC antigen expression in the presence of potent inducers, such as IFN-y.

ANTAGONISM OF TIFN-Y-INDUCED IA ANTIGEN EXPRESSION

Many substances have been demonstrated to antagonize the induction of class II MHC antigen expression by IFN-γ. These include: prostaglandins of the E series (PGE's; Snyder et al., 1982; Kelley and Roths, 1982), analogs of adenosine 3'-5'-cyclic monophosphate (cAMP; Yem and Parmely, 1981; Steeg et al., 1982b; Hanaumi et al., 1984), lipopolysaccharide (LPS; Yem and Parmely, 1981; Steeg et al., 1982b; Koerner et al., 1987; Vermeulen et al., 1987), immune complexes (Hanaumi et al., 1984; Virgin et al., 1985), α-fetoprotein (Lu et al., 1984), serotonin (Sternberg et al., 1986), norepinephrine (Frohman et al., 1988), glucocorticoids (Snyder and Unanue, 1982; Warren and Vogel, 1985b), and IFN-α/β (Ling et al., 1985; Inaba et al., 1986). Most of the studies of the antagonism of IFN-y-induced class II MHC expression have been performed at a protein level. Work by Kelley and Roths (1982) have shown that peritoneal macrophages isolated from PGE-treated, autoimmune MRL-lpr mice have significantly reduced levels of cell surface Ia antigen in comparison to untreated control mice (which express aberrantly high levels of Ia antigen). Several in vitro studies which analyzed the effects of PGEs on Ia antigen expression in macrophages have shown that these arachidonic acid metabolites, when added to cultures exogenously, induce the intracellular

accumulation of cAMP, which in turn, can lead to the suppression of Ia antigen expression (Snyder et al., 1982; Steeg et al., 1982b; Tripp et al., 1986). The role of endogenous PGEs has been difficult to address given the lability of these metabolites (Tripp et al., 1986). However, it has been suggested by Tripp et al. (1986) that the inverse relationship between PGE synthesis and Ia antigen expression may imply that the production of endogenous PGEs during inflammation plays a role in the termination of a normal immune response. Steeg et al. (1982b) demonstrated that the ability of LPS to antagonize IFN- γ -induced Ia antigen expression is counteracted by the presence of the cyclooxygenase inhibitor, indomethacin. In addition, exogenously added PGE and dibutryl cAMP were effective antagonists of IFN- γ -induced cell surface Ia antigen. Given the observation that PGE-treated macrophages exhibit elevated levels of cAMP (Gemsa et al., 1975; Bonta et al., 1981; Gemsa, 1981), Steeg et al. (1982b) proposed that the antagonism of Ia antigen induction by LPS is mediated through the production of PGEs, which, in turn, lead to increased levels of cAMP.

Several mechanisms have been postulated for immune complex-mediated antagonism of Ia antigen expression. These include: the induction of prostaglandins and subsequent production of cAMP, the direct activation of adenylate cyclase, the reduction in the number of IFN- γ receptors, or the production of a distinct second messenger (i.e., DAG and IP₃). Nitta and Suzuki (1982) have shown that the binding of immune complexes to either class of Fc γ receptors on P388D₁ (i.e., Fc γ _{2a}R or Fc γ _{2b}R) results in the synthesis of cAMP. Furthermore, they demonstrated that the accumulation of cAMP in IgG_{2a} immune complex-treated cells was very rapid (i.e., within 30 min of treatment) whereas, the accumulation of cAMP in IgG_{2b} immune-complex-treated cells was gradual and prolonged (lasting for > 6 hr post-treatment). Fc γ _{2a}R-stimulated synthesis of cAMP was shown to be insensitive to the effects of inhibitors of either the phospholipase A₂ or the cyclooxygenase pathway and the rapid nature of cAMP accumulation supports the hypothesis that stimulation of this receptor results in the direct activation of an adenylate

cyclase. More recent evidence from Hirata and Suzuki (1987) demonstrated a casein kinase activity associated with the Fcy2aR isolated from the macrophage cell line P388D1. Since the receptor itself is an acidic protein and the casein kinase has a strong preference for acidic substrates, it is possible that the receptor undergoes autophosphorylation following IgG_{2a} binding. On the other hand, the $Fc\gamma_{2b}R$ has been shown to possess an intrinsic phospholipase A2 activity (Suzuki et al., 1982) and, consistent with this finding, is the observation that phospholipase A2 and cyclooxygenase inhibitors abrogate the ability of IgG_{2b} immune-complex-treated cells to synthesize cAMP. These observations, taken together with the delayed accumulation of cAMP in cells stimulated by IgG2b complexes, support the hypothesis that stimulation of the Fcy2bR results in the production of prostaglandins which, in turn, activate adenylate cyclase to increase intracellular levels of cAMP. Hanaumi et al. (1984) have extended this work and have shown that the ability of immune complexes that bind specifically to IgG_{2b} receptors to suppress IFN-γ-induced Ia antigen expression is abrogated in the presence of inhibitors of either the phospholipase A2 or the cyclooxygenase pathway. However, these inhibitors were shown to have no effect on the ability of immune complexes specific for IgG_{2a} receptors to suppress IFN-γinduced Ia antigen expression. The exact nature of the modulation of IFN-γ-induced Ia antigen expression by cAMP is not yet clear.

The antagonist α -fetoprotein appears to modulate Ia antigen expression through a mechanism which is independent of PGE production (Lu et al., 1984). This mechanism has not yet been elucidated but has been hypothesized to be operational during the neonatal period when the organism "learns" to be tolerant to self-antigens that are newly expressed on differentiating tissue (Lu et al., 1984). Inaba et al. (1986) demonstrated that a similar neonatal hyporesponsiveness, i.e., the failure of IFN- γ to induce Ia antigen expression in newborn macrophages, could be reversed by the addition of anti-IFN- β monoclonal antibody. These findings suggest that the presence of certain antagonists of Ia antigen expression in the newborn may account for their relatively poor capacity to respond to

foreign, as well as self-antigens.

Serotonin, a platelet-derived inflammatory mediator and neurotransmitter, has been shown to suppress IFN-γ-induced macrophage Ia antigen expression following its binding to the serotonin receptor (Sternberg et al., 1986). The second messenger(s) that is (are) triggered following this transmitter-receptor interaction is (are) unknown. Recent evidence has shown that another neurotransmitter, norepinephrine (NE), inhibits IFN-γ-induced class II MHC expression on brain astrocytes (Frohman et al., 1988). It has been suggested that the astrocyte is one of the major antigen-presenting cells in the brain and the finding that NE antagonizes IFN-γ-induced Ia antigen expression may represent a method by which immune responsiveness in the central nervous system is negatively controlled (Frohman et al., 1988). Mechanistically, it has been proposed (from preliminary experiments) that NE may exert its antagonistic effect through β-adrenergic receptors which have been shown previously to stimulate the formation of cAMP (Hansson et al., 1985; McCarthy and de Vellis, 1978; Van Calker et al., 1978).

Glucocorticoids have been shown to operate as transcriptional modulators of gene activity in a number of systems which include Mouse Mammary Tumor Virus (Ringold et al., 1975; Ringold, 1983; Payvar et al., 1983), metallothionein I and II genes (Hager and Palmiter, 1981; Karin et al., 1984), growth hormone gene (Spindler et al., 1982), and β-globin gene (Mierendorf and Mueller, 1982). In addition, DEX has been recently shown to depress mRNA levels for Interleukin 3 (IL 3; Culpepper and Lee, 1985), T cell growth factor (IL 2; Arya et al., 1984), and IFN-γ (Arya et al., 1984). The mechanism by which glucocorticoids and other steroid hormones modulate gene activity is believed to occur via a two-step model which involves: (i) binding of the glucocorticoid (or hormone) to a soluble intracellular receptor to form an "activated" complex which has increased affinity for interphase chromosomes, and (ii) subsequent interaction of this "activated" hormone-receptor complex with specific DNA sequences. This results in the modulation of transcription of a proximal gene (Yamamoto, 1985). The role of this two-step mechanism

in the ability of glucocorticoids to down-modulate IFN-γ-induced Ia antigen expression is unclear. The action of potential second messengers (i.e., an "activated" steroid-receptor complex) and glucocorticoid-responsive DNA regions upstream of the Ia genes have not yet been demonstrated. However, this antagonism has been shown to be independent of arachidonic acid metabolism, as evidenced by the failure of phospholipase A2 antagonists or other inhibitors of the cyclooxygenase pathway to block the down-regulation of IFN-γ-induced Ia antigen expression induced by DEX (Warren and Vogel, 1985). The recent finding that 1,25(OH)2D3 treatment causes a reduction in class II MHC gene expression of a melanoma cell line (both in terms of protein and message) may be indicative of a down-regulatory mechanism similar to that of DEX (Carrington et al., 1988). The steroid hormone, 1,25(OH)₂D₃ plays a critical role in the maintenance of calcium and phosphorus homeostasis (DeLuca, 1986) and, as a member of the steroid hormone family, it interacts specifically with a soluble nuclear receptor to form a complex which has an increased binding affinity for specific DNA regions (DeLuca, 1986). Potential candidates for DNA targets include those genes that code for proteins involved in calcium transport and a calcium binding protein which has been shown to be 1,25(OH)2D3-dependent (Bishop et al., 1984). There are two reports which demonstrate that glucocorticoid treatment of chick fibroblasts (Krishnan and Baglioni, 1980) and lymphoblastoid cells (Oikarinen, 1982) results in increased levels of 2',5'-oligoadenylate synthetase. In addition, IFN-α, -β, and -γ are considered to be principal inducers of 2',5'- oligoadenylate synthetase in many experimental systems (reviewed by Johnston and Torrence, 1984). The ability of 2',5'-oligoadenylate synthetase to activate a 2',5'- oligoadenylate-dependent endoribonuclease has been established (reviewed by Johnston and Torrence, 1984); however, the capacity of this endoribonuclease to degrade RNA species discriminately has not been demonstrated (Clemens and William, 1978; Schmidt et al., 1978). Although treatment with IFN-α/β and DEX did not result in gross degradation of rIFN-γ-induced A_α-specific mRNA, as assessed by Northern blot analysis, one would need to preform

more rigorous experiments addressing the issue of message stability to determine the role, if any, of the 2',5'-oligoadenylate system in message degradation.

Lastly, the action of IFN- α/β in the down-modulation of IFN- γ -induced Ia antigen expression in murine macrophages has also been shown to be independent of arachidonic acid metabolism, as evidenced by the ability of IFN- α/β to antagonize the induction of Ia antigen expression in the presence of phospholipase A2 antagonists or other inhibitors of the cyclooxygenase pathway (Ling et al., 1985). However, the role of second messengers involved in these systems have not yet been elucidated. With regard to the antagonism of Ia antigen induction on murine macrophages by IFN-α/β, down-regulation of IFN-y receptors does not appear to be a contributing factor (Dr. D. Finbloom, personal communication). IFN-\alpha has been shown to alter directly gene activity at the level of transcription in several systems, including 2',5'-oligoadenylate synthetase (Krishnan and Baglioni, 1980), metallothionein-II (Friedman and Stark, 1985), and class I MHC antigens (Friedman and Stark, 1985; Israel et al., 1986). It is also interesting to note that IFN-α/β has been shown to antagonize the expression of two other IFN-y-induced macrophage membrane markers, i.e., the mannosyl-fucosyl receptor and the tumor necrosis factor receptor, although nothing is known about the effect of IFN-α/β on the mRNA for these products. Active investigation is ongoing to establish the mechanisms by which these antagonists of Ia antigen expression act with the hope of employing these agents therapeutically to treat certain autoimmune states.

GOALS OF THIS DISSERTATION

The goals of this dissertation were to examine molecular mechanisms which underlie the induction of Ia antigen on macrophages by rIFN- γ and to elucidate how this induction process is antagonized by the selected inhibitors, IFN- α/β and DEX. In

choosing an appropriate system in which to study these phenomena, the following considerations were taken into account: (i) selection of a macrophage culture system which accurately represents the heterogeneity of macrophage populations as they exist in vivo, and (ii) choice of an appropriate mouse strain whose macrophages are sensitive to both the inducer and the inhibitors. With regard to these considerations, the C3H/HeJ mouse strain was selected. Although C3H/HeJ mice are hyporesponsive to Gram negative endotoxin (LPS), their macrophages are fully capable of being activated in vitro by a number of agents. For example, these macrophages have been shown to respond to IFN-y with both increased Fc receptor and Ia antigen expression (Vogel et al., 1982; Fertsch and Vogel, 1984; Warren and Vogel, 1985; Ling et al., 1985). With respect to the number of cells expressing Ia antigen, < 5% of thioglycollate-elicited, peritoneal exudate macrophages have detectable basal levels of Ia antigen; however, > 40% of these macrophages can be induced to express Ia antigen following treatment with IFN-γ (Warren and Vogel, 1985b; Ling et al., 1985). The ability of IFN-y to induce cell surface Ia antigen expression in macrophages has been analyzed and confirmed in vivo and in vitro, using a variety of mouse strains and cell lines and recombinant preparations of murine IFN-γ (King and Jones, 1983; Zlotnick et al., 1983; Nakamura et al., 1984; Skoskiewicz et al., 1985). Previous work had also revealed that Ia antigen expression on IFN-γ-induced C3H/HeJ macrophages is highly sensitive to modulation by IFN-α/β (Ling et al., 1985) and DEX (Warren and Vogel, 1985b). These results were confirmed prior to initiation of the molecular aspects of this study (Table 1). The results in Table 1 corroborate previous findings which demonstrate the sensitivity of the C3H/HeJ macrophage system in an examination of the induction of Ia antigen expression by IFN-γ and its down-regulation by IFN- α/β and DEX. To demonstrate that the results obtained from the LPS-hyporesponsive macrophages were representative of other mouse strains (e.g., those that are genetically LPS-responsive), experiments were also performed to examine Ia antigen cell surface expression, as well as the levels of Ia-specific RNA, in macrophages from fully

LPS-responsive, C3H/OuJ mice. Although a number of other studies have employed macrophage-like cell lines to examine Ia expression, primary macrophage cultures were selected for these studies because they are more representative of macrophage populations found in nature, both in terms of their heterogeneity and mortality.

STEADY-STATE ANALYSIS OF IA GENE EXPRESSION

In the studies presented herein, initial experiments were designed to confirm and extend previous findings of induction and down-regulation of Ia antigen expression at a protein level (Warren and Vogel, 1985b; Ling et al., 1985). Table 1 illustrates the induction of Ia antigen expression on macrophages with IFN-y (5.0 U/ml) and its down-regulation by IFN- α/β (100 U/ml) and DEX (1 x 10⁻⁵M). In addition, the inclusion of R5020 (a pure progestin) and the demonstration that it failed to modulate IFN-y-induced Ia antigen expression, confirms that the negative effect of glucocorticoids on IFN-γ-induced Ia antigen expression is mediated specifically through their interaction with the glucocorticoid receptor. Although the analysis of Ia antigen expression at a protein level has been extensive (reviewed in Unanue, 1984; Wong and Schrader, 1985; Warren and Vogel, 1985b; Ling et al., 1985; Inaba et al., 1986), limited studies have been performed to elucidate the molecular mechanisms that underlie the induction and antagonism of Ia antigen expression. Early work by Nakamura et al. (1984), demonstrated that IFN-y treatment of P388D1 cells (a macrophage-like cell line) led to a significant increase in I region mRNA levels. Studies by Paulnock-King et al. (1985) revealed that IFN-γ mediates the coordinate induction of mRNA for several I region loci in another macrophage-like cell line, WEHI-3. Similarly, Wake and Flavell (1985) demonstrated the accumulation of A_{B1} mRNA after treatment of peritoneal exudate macrophages and J774A.1 cells (a macrophage-like cell line).

In the present studies, steady-state levels of Ia mRNA were first analyzed in response to rIFN-γ treatment in an attempt to establish a model primary macrophage culture system with which the molecular mechanisms involved in induction and antagonism of Ia antigen expression might be elucidated. To examine the induction quantitatively, the expression of the DHFR gene was also monitored and was used subsequently as an internal control standard since its expression remained constant with the treatments tested and over the time course studied (Figure 4). Five to 10 U/ml rIFN-y induced maximal levels of A_r-specific RNA (Table 3) and these concentrations were comparable to those found previously to induce maximal cell surface expression of Ia antigen in a similar culture system (Warren and Vogel, 1985; Ling et al., 1985; Vogel et al., 1986). This optimal concentration of rIFN-y caused a 5.7 to 6.5-fold increase in steady-state levels of total or cytoplasmic A_C-specific RNA (Figure 3 and Table 2). Treatment of macrophages with rIFN-y resulted in the dose-dependent, induction of mRNA for multiple I-region loci, including A_{α} , A_{β} , and E_{α} , consistent with coordinate induction which was reported previously for the WEHI-3 cell line (Paulnock-King et al., 1985). However, since we did not carry out time-course studies for the accumulation of A_{β} and E_{α} steady-state RNA species, we cannot be certain that the kinetics of accumulation of steady-state levels of these mRNAs are the same as those for A_{α} -specific mRNA. Dose-dependent induction of steady-state levels of A_{α} -specific RNA following IFN- γ treatment was also observed in macrophages from C3H/OuJ mice (LPS-responders; Table 8), and demonstrates that induction of Ia antigen expression by rIFN-γ is not restricted to LPS- hyporesponsive, C3H/HeJ macrophages. Removal of rIFN-y at any time during the 24 hr induction period resulted in decreased accumulation of A_α-specific mRNA (Figure 6). This finding suggests that rIFN-γ does not merely trigger the induction process, but that its sustained presence is necessary for the induction of maximal levels of steady-state, Aq-specific RNA. Addition of CHX at various times after treatment of cultures with rIFN-y revealed that the capacity to produce protein during the first 12 hr of this induction period is critical

to the accumulation of maximal levels of A_{α} -specific RNA (Figure 7). The requirement of protein synthesis for the maximal induction of Ia gene expression by rIFN-γ is consistent with the relatively long induction phase (Figure 3 and Table 2) and with a previous report by Walker et al. (1984). Their data suggested the existence of an intermediate, trans-activating factor secreted by IFN-γ-treated macrophages which confers Ia-inducing activity to other macrophages in the apparent absence of IFN-y. Lastly, data from Zuckerman et al. (1988) have also provided evidence in support of the involvement of a trans-activating factor(s) in the induction of Ia antigen by IFN-γ. They showed that the murine macrophage cell line, PU5, which is incapable of constitutive or IFN-y-inducible expression of Ia antigen, could be rendered inducible with IFN-y following fusion with a peritoneal exudate macrophage that putatively supplies a trans-activating factor. This finding suggests that the defect in the unfused PU5 cell line resides in the locus for the trans-activating factor and not the I-region structural genes. In addition, they demonstrated that there was no difference in the number of IFN-y receptors in the fusion partners or in the resulting hybrid. These observations, taken together with the lag time observed prior to the induction of Ia mRNA and the CHX sensitivity of Ia mRNA accumulation presented herein, support the hypothesis that IFN-y induces Ia antigen expression through an indirect mechanism which involves the production and/or action of an intermediate protein. One possibility is that IFN-y treatment results in the synthesis of an intermediate, transactivating protein, which in turn, increases transcription of class II MHC genes. An alternative mechanism might involve the antagonism of the production and/or action of a factor that normally suppresses class II MHC gene expression which, in turn, would lead to increased transcription of class II MHC genes. Lastly, evidence for a trans-acting factor in the regulation of class II MHC gene expression comes from studies of peripheral blood lymphocytes from patients with congenital severe combined immunodeficiency disease (dé Preval et al., 1985). Lymphocytes from these patients fail to express any class II MHC antigens. The inability to express class II MHC antigens could not be reversed by the

addition of IFN- γ , even though the lymphocytes apparently expressed IFN- γ receptors. It has been postulated by dé Preval <u>et al</u>. (1985) that this global inability to express class II MHC antigens may be due to a defect in a trans-activating gene, unlinked to the MHC, that is involved in the regulation of these antigens.

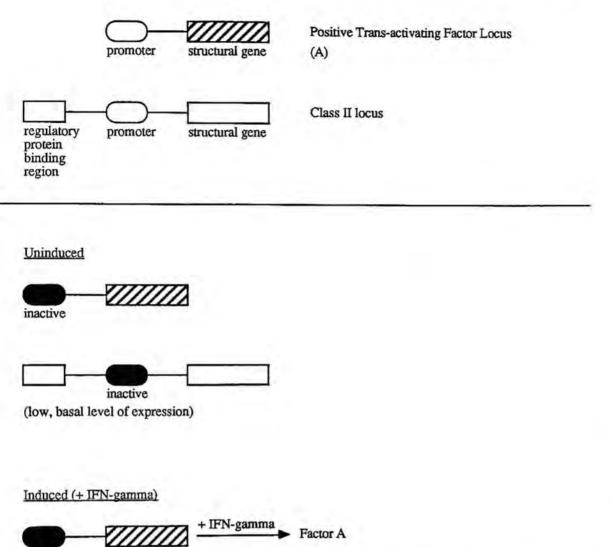
One potential candidate for the trans-activating, Ia-inducing factor is an IFN-γ-induced secretory protein, IP-10, described recently by Luster and Ravetch (1987). The protein is the product of a gene whose mRNA is detectable in human mononuclear cells as early as 30 min after IFN-y treatment (Luster, 1987; Luster and Ravetch, 1987). Based on amino acid homolgy, the product of this gene appears to belong to a family of chemotactic and mitogenic proteins associated with inflammation and proliferation. Kaplan et al. (1987) have extended this work using in situ immunocytochemistry with antibodies directed against IP-10 and class II MHC antigens and demonstrated the cellular co-localization of IP-10 and Ia antigens in tissue sections of skin from lepromatous patients which were sensitized previously with either purified protein derivative (PPD) or rIFN-y. Eighteen hr following the injection of PPD or rIFN-y, a greater proportion of keratinocytes stained for IP-10 than for Ia antigen which suggests that IP-10 synthesis precedes Ia antigen synthesis in this delayed-type hypersensitivity environment. The kinetics of induction, the co-localization of these two proteins, and the cytoplasmic staining patterns of IP-10 are consistent with the hypothesis that IP-10 may represent the putative, trans-activating, Ia-inducing factor.

The steady-state RNA experiments presented herein have raised some interesting questions concerning the regulation of basal and induced levels of Ia antigen expression. The Northern blot in Figure 3 reveals that even in the absence of IFN- γ , low but detectable steady-state levels of A_{α} -specific RNA exist. This suggests that the A_{α} -locus is transcribed, even in untreated macrophages, albeit to a lesser extent. Several models that might account for these observations, as well as the lag time in induction and CHX sensitivity of mRNA accumulation, are presented below and in Figures 21 and 22. One

model, the "activator" model (Figure 21), proposes that a trans-activating factor (Factor A), induced by treatment with IFN-γ, interacts directly with DNA regulatory regions upstream of class II MHC antigen structural genes. Binding of Factor A to this region of DNA facilitates the binding of RNA polymermase II to the promoter. In the absence of the trans-activating factor, transcription proceeds, but at a greatly reduced level due to the unfavorable conformation of the DNA at the RNA polymerase II binding site. Another model, the "repressor-displacement" model (Figure 22), is a variation of the "activator" model. In this model, Factor A competes with an endogenously-produced repressor factor (Factor R) for binding to a DNA regulatory region upstream of the class II MHC antigen structural genes. In the absence of IFN-y, Factor R binds to the DNA such that the resulting conformation is not favorable for RNA polymerase II binding at this site. Transcription which results from limited RNA polymerase II binding under these conditions results in basal steady-state levels of Ia gene expression (i.e., those levels observed in untreated macrophage cultures). Following IFN-y treatment, Factor A accumulates and competitively displaces Factor R. The promoter region is now in a favorable conformation for RNA polymerase II binding. Transcription that results from this enhanced RNA polymerase II binding is reflected by induced levels of Ia gene expression (i.e., those steady-state levels observed in IFN-γ-treated macrophage cultures). Another model which is also completely consistent with the observations is one similar to the "repressor-displacement" model with the modification that Factor A prevents the synthesis of Factor R, rather than sterically hindering the binding of Factor R to the regulatory region. From the steady-state induction data presented thus far, it seems likely that the putative trans-activating factor, which is necessary for the induction of Ia antigen, facilitates RNA polymerase II binding either through the production of a positive effector (i.e., an activator) or through the antagonism of a negative effector (i.e., a repressor).

With the establishment of an <u>in vitro</u> system for the study of the accumulation of steady-state levels of Ia mRNA in response to rIFN-y, the next goal was to examine the

Figure 21. Representation of the "activator" model of class II MHC gene expression. In this model it is proposed that IFN-γ treatment of macrophages leads to the production of a transactivating factor. The binding of this factor to a region upstream of the class II MHC gene promoter leads to increased transcription of class II structural genes. The shaded promoter ovals indicate regions of low, basal transcriptional activity while the unshaded promoter ovals indicate regions of high, induced transcriptional activity. "A" is an activator molecule.



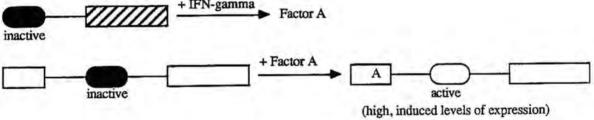
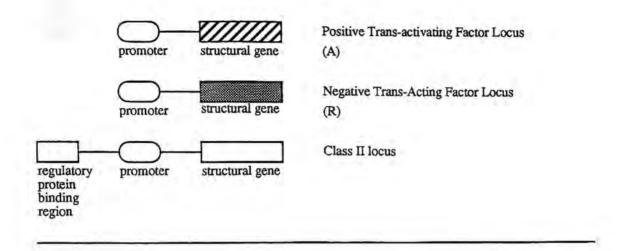
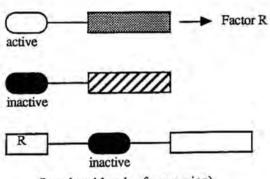


Figure 22. Representation of the "repressor-displacement" model of class II MHC gene expression. In this model, treatment of macrophages with IFN-γ leads to the production of a trans-activating factor. However, in contrast to the model shown in Figure 21, this factor displaces a repressor (which is bound in the uninduced state) and leads to increased transcription of class II structural genes. The shaded promoter ovals indicate regions of low, basal transcriptional activity while the unshaded promoter ovals indicate regions of high, induced transcriptional activity. "A" is an activator molecule and "R" is a repressor molecule.

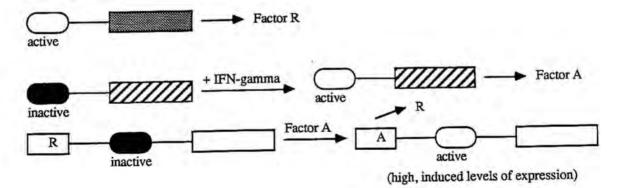


Uninduced



(low, basal levels of expression)





steady-state levels of Ia mRNA following treatment with IFN- γ in the presence of either IFN- α/β or DEX. The data in Table 1 serve to confirm previously reported findings of the antagonism of IFN- γ -induced Ia antigen expression (i.e., at a protein level) by IFN- α/β (Ling et al., 1985) and DEX (Warren and Vogel, 1985b) on cultures of peritoneal exudate macrophages. The data illustrate that treatment of macrophages with rIFN- γ in the presence of either IFN- α/β or DEX, leads to a significant reduction in cell surface Ia antigen (as detected in the ELISA assay). As described in Materials and Methods, these experiments were carried out in the presence of media which was supplemented with 2% FCS. The possibility arose that steroidal and non-steroidal hormones present in the FCS supplement might modulate Ia antigen expression. This possibility was addressed in experiments using a serum-free, compositionally-defined media supplement, HL-1 (Vogel et al., 1988). The results of these studies indicated that the induction of Ia antigen expression by rIFN- γ in either FCS- or HL-1-supplemented media, as well as down-regulation of Ia expression by DEX, was comparable.

The protein profiles of Ia antigen antagonism were extended next to an analysis of steady-state RNA levels. Both IFN- α/β (Figures 9 and 10 and Table 6) and DEX (Figures 12 and 13 and Table 7) reduced significantly, in a dose-dependent fashion, the levels of A_{α} - (as well as A_{β} - and E_{α} -) specific mRNA induced by rIFN- γ . The concentrations of IFN- α/β (10 - 100 U/ml) and DEX (5 x 10⁻⁶ - 1 x 10⁻⁵M) that resulted in maximal reductions in Ia-specific RNA were chosen based on their ability to antagonize rIFN- γ -induced Ia antigen expression in previous protein studies and are well within the upper physiological levels achievable therapeutically (Cantell and Pyhälä, 1973; Cantell et al., 1974; Guyre and Munck, 1986). It is also probable that inhibitory levels of IFN- α/β and DEX are achievable in local inflammatory environments (Gresser, 1961; Roberts et al., 1979; Shek and Sabiston, 1983; Besedovsky et al., 1975; 1986). The magnitude of this inhibition was most striking when suboptimal doses (0.5 U/ml) of rIFN- γ were used, and these findings strongly parallel those reported previously at a protein level. Comparable

results were observed in macrophages from C3H/OuJ mice, again demonstrating that the antagonism of rIFN-γ-induced Ia antigen expression by IFN-α/β and DEX is not unique to macrophages from LPS-hyporesponsive C3H/HeJ mice. Since the reduction in cell surface expression of Ia antigen can be related to a reduction in steady-state levels of Ia mRNA, it was hypothesized that the specific inhibitors, IFN-α/β and DEX, act pretranslationally to reduce levels of rIFN-y-induced, steady-state Ia mRNA. However, from these studies one cannot rule out the possibility that IFN- α/β and DEX may also play a role in post-transcriptional events which result in reduced steady-state levels of Ia mRNA as well as reduced cell surface Ia antigen expression (i.e., destabilization of Ia message or a block in the translation of Ia message). The inhibition of maximal accumulation of rIFN-γ-induced, steady-state A_α-specific mRNA levels observed in the presence of IFN- α/β and DEX could result from: (i) the destabilization of Ia mRNA, (ii) the antagonism or destabilization of an inducible, trans-activating factor, (iii) the production or destabilization of a constitutively expressed repressor factor, or (iv) a direct blockade of the I-region locus. Previous findings that pretreatment of macrophages with IFN-α/β or DEX (prior to treatment with IFN- γ) or addition of IFN- α/β or DEX early in the induction phase led to a significant inhibition of Ia antigen expression at a protein level (Ling et al., 1985; Warren and Vogel, 1985b), might suggest that for the inhibitors to antagonize the IFN-y-mediated induction, they must be added prior to the period of class II MHC gene transcription initiation or prior to the production of the putative trans-activating protein. The additional finding that the inhibitory effects of DEX are most striking when the inhibitor is added 2 hr after IFN-γ, also suggests that DEX may be acting at some early stage in the induction phase to inhibit IFN-y-induced Ia antigen expression. For these reasons, IFN-α/β and DEX may exert their inhibitory effects indirectly, rather than by physically blocking the interaction of RNA polymerase II with I-region DNA regulatory regions. If IFN-α/β and DEX interacted with I-region DNA regulatory regions, one might expect to see inhibition of IFN-y-induced Ia protein expression even if the inhibitors were

added late in the culture period (i.e., between 24 and 48 hr). This is not observed.

To date, most of the analysis of the down-regulation of IFN-γ-induced Ia antigen expression by specific inhibitors has been performed at a protein level. Concurrent with the publication of our results of the steady-state analysis (Fertsch et al., 1987), Koerner et al. (1987) published an analysis of the inhibitory effects of LPS on IFN-γ-induced Ia mRNA accumulation. Briefly, their work revealed that LPS treatment leads to a 50 - 80% suppression in the accumulation of A_B-specific mRNA. The magnitude of this suppression is similar to that seen for IFN- α/β (Figures 9 and 10) and DEX (Figures 12 and 13). The ability of LPS to down-regulate IFN-γ-induced A_β-specific mRNA was shown to require protein synthesis. LPS was also an effective antagonist when present early in the induction period (i.e, during the first 12 hr). Similarly, Vermeulen et al. (1987) demonstrated that LPS antagonized the ability of IFN-γ to induce HLA-DR mRNA in a human macrophage system. The role of the proposed second messengers (i.e., PGE, cAMP, DAG, and IP₃) induced by LPS treatment in the suppression of IFN-y-induced, steady-state levels of I-region RNA has not been demonstrated. However, the recent isolation and characterization of six LPS-induced genes from a cDNA library of LPS-treated peritoneal macrophages (Tannenbaum et al., 1988) may allow for the elucidation of the intermediate factors involved in LPS-induced alterations in Ia antigen expression.

Given the findings that IFN-α/β and DEX result in a significant decrease in the accumulation of rIFN-γ-induced, Ia mRNA (Figures 9, 10, Table 6 and Figures 12, 13, Table 7, respectively), further experiments were performed to compare nuclear and cytoplasmic RNA preparations from inducer- plus antagonist-treated macrophage cultures. The rationale for examining these distinct populations of RNA was to determine if the inhibitors acted by modulating the transport of Ia mRNA across the nuclear membrane into the cytoplasm. If this were the case, then one would expect to observe comparable basal and rIFN-γ-induced levels of Ia mRNA in nuclear and cytoplasmic preparations but a significant reduction in the accumulation of Ia cytoplasmic mRNA in those cultures which

had been treated with IFN- γ plus IFN- α/β or DEX. The results shown in Figure 14 indicate that the patterns of induction and down-regulation are similar in both nuclear and cytoplasmic RNA preparations. This finding suggests that the reduction in steady-state levels of A_{α} -specific RNA is not the result of an alteration in the rate of transport of A_{α} -specific mRNA from the nucleus to the cytoplasm.

Preliminary experiments were also performed to address the possibility that the reduction in steady-state levels of Ia mRNA may be the result of a mechanism which causes an accelerated degradation of mRNA. To test this hypothesis, macrophage cultures were treated for 24 hr with rIFN- γ to induce the accumulation of Ia mRNA, at which time the cells were washed extensively and actinomycin D (Act D) was added to block new RNA synthesis. Cytoplasmic RNA was harvested at various time intervals thereafter. Difficulties arose in the interpretation of the data due to the observation that the A_{α} -specific mRNA levels remained elevated, without any significant indication of decay, for > 12 hr in the absence or presence of the inhibitors. Given that the half-life of the A_{α} -specific mRNA appears to be relatively long, coupled with the observation that the concentration of Act D required to reduce new mRNA synthesis by > 90% was toxic with time in culture (> 12 hr), it was not possible to evaluate the potential role of IFN- α / β and DEX in the acceleration of the decay of A_{α} -specific mRNA.

TRANSCRIPTION RATE ANALYSIS OF IA GENE EXPRESSION

Taken collectively, the results of the steady-state experiments described above led us to hypothesize that the positive and negative regulation of Ia antigen expression by IFN-γ and the inhibitors might be controlled by modulating the rate at which the class II MHC antigen genes were transcribed. To address directly the validity of this hypothesis, in vitro transcription elongation studies were undertaken. These experiments provide a

relative measure of the number of RNA polymerase II molecules initiated on the gene under study. Subsequently, an in vitro transcription assay system was developed to insure strand-specificity (Figure 17), the dependence on the activity of RNA polymerase II (Table 9), the optimization of length of hybridization period (Figure 16), and the presence of sufficient excess of cDNA immobilized onto filters to ensure linear, quantitative results (Figure 15). Initially, transcription elongation assays were performed on nuclei isolated from macrophage cultures treated with IFN-y only. These experiments demonstrated that rIFN-y increases the rate of I-region gene transcription 3.7-fold, or more specifically, that rIFN-y induces a 3.7-fold increase in the levels of I-region transcripts initiated prior to their elongation in vitro (Figure 19). The increase in the rate of I-region gene transcription was maximal 24 hr post-induction with rIFN-y. The decrease in the rate of I-region gene transcription observed at 36 and 48 hr might reflect the degradation of rIFN-y in the culture medium. These transcription findings are consistent with the time course data (Figure 3). The sustained magnitude of the accumulation of RNA reported for the steady-state analysis (Table 2) may reflect the long half-life of Ia-specific mRNA, even though the rate of transcription declines after 24 hr (Figure 19).

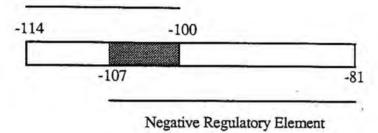
In addition to the results of the steady-state and transcription elongation experiments presented above, other investigators have concluded from gene transfer studies (i.e., by transfection and transgenic manipulations) that transcription plays a critical role in the induction of class II gene expression by IFN- γ . Transfection studies, in which plasmids that contain selected deletions of the 5' regulatory region of HLA-DR $_{\alpha}$ were introduced into a glioblastoma cell line (Basta et al., 1987), have led to the identification of a DNA sequence that is necessary for the inductive effects of IFN- γ . Within this sequence a nonamer, AGAAGN(A/C)AG, was identified that appeared to be conserved in the 5' flanking regulatory regions of other IFN- γ -inducible genes [i.e., other human class II MHC antigen genes (HLA-DR $_{\beta}$), a human class I MHC antigen gene (HLA-A2), several murine class II MHC antigen genes (I-A $_{\beta}$ ^b, I-E $_{\beta}$ ^b, and I-E $_{\beta}$ ^b), two murine class I MHC

antigen genes (H-2 K^k and H-2 K^b), and β -2 microglobulin]. One might postulate that this sequence binds an IFN- γ -induced trans-activating factor and facilitates RNA polymerase II binding. Thus, one might predict that a common trans-activating factor may be directly responsible for the induction of a variety of IFN- γ -inducible proteins.

Similarly, transfection studies (Boss and Strominger, 1986) of 5' regulatory sequences of HLA-DQB (and selected deletions within this region) into cell lines have led to the identification of: (i) two positive elements, which are required for the high levels of expression, one from a region -114 to -100 bp (upstream of the cap site) and another from a region -80 to -67 bp (upstream of the cap site), and (ii) two negative regulatory elements, which may play a role in the suppression of class II MHC antigen epression in cells that normally do not present antigen and in uninduced APCs, one from a region 5' from -128 bp (upstream of the cap site) and another from a region -107 to -81 bp (upstream of the cap site). The overlap of the positive and negative elements, between positions -107 and -100, suggests a potential mechanism whereby binding of a trans-activating factor, that is induced by IFN-y, could actually displace or compete for binding with an endogenous repressor that normally binds to the negative element (Figure 23). Precedent for such a model has recently been established in the regulation of IFN-β gene expression (Goodbourn and Maniatis, 1988). These investigators have shown that within the IFN-β gene regulatory region (5' upstream of the IFN-β structural gene), there are distinct, but overlapping, positive and negative regulatory domains. An analysis of constructs which contain mutations in this overlap region suggest the following: (i) a repressor may bind to the negative element in the uninduced state and subsequently prevent the binding of transcription-promoting factors to the positive element; (ii) transcription-promoting factors in uninduced cells may bind to the positive element and result in basal levels of gene expression; and, (iii) synthesis of positive transcription factors following induction may displace the bound repressor from the negative element and result in induced levels of gene expression. The functional significance of the overlap of positive and negative regulatory

Figure 23. Representation of the potential regulatory elements involved in class II MHC gene expression. Elements involved in the regulation of the human class II MHC gene, HLA-DQ β have been described by Boss and Strominger (1986). Two of these elements have a region of overlap which may be involved in the activation of this gene by IFN- γ .

Postive Regulatory Element



regions 5' upstream of the DQ β gene awaits the results of similar mutational analyses.

Lastly, microinjection studies (Dorn et al., 1987a) of E_{α} gene constructs with selected deletions of 5' regulatory regions into embryos derived from mice incapable of normally expressing E_{α} genes have lead to the identification of two invariant sequence elements, the X box (-93 to -80 bp upstream of the cap site) and the Y box (-61 to -48 bp upstream of the cap site). Analysis of deletion constructs of these boxes have revealed some interesting observations which include: (i) the X and Y box sequences are involved in the efficiency and accuracy of transcription initiation; (ii) the Y box sequence is critical for the induction of class II MHC antigen expression by IFN-y, and (iii) the Y box sequence also contains a CCAAT sequence in reverse (ATTGG). Using gel electrophoresis retardation methods, which allow for the identification of specific DNA-binding proteins based on altered electrophoretic mobility patterns of protein-DNA complexes, Dorn et al. (1987a) have identified a nuclear factor, NF-Y, that binds to the Y box of the E_{\alpha} gene. In addition, NF-Y was shown to bind to CCAAT sequences of a number of unrelated genes, albeit with less affinity than that of the E_{α} Y-box (Dorn et al., 1987b). Given the rather generalized nature of the binding of NF-Y to other CCAAT boxes, it is probable that other accessory proteins are needed to confer specificity to the regulation of Eq. gene transcription. Celada et al. (1988) have performed similar experiments using nuclear extracts from a variety of tumor cell lines including B cells, T cell, macrophages, mastocytes, and fibroblasts to analyze nuclear proteins interactions with DNA sequences upstream of the coding region of the AB gene. Nuclear extracts from all these cell lines caused an identical electrophoretic retardation pattern of an oligonucleotide which contained the AB Y-box sequence. In addition, nuclear extracts isolated from IFN-γ-treated macrophages failed to exhibit any additional effect on the binding activity than nuclear extracts from untreated, control macrophages. Given the localization of this Y-box-binding protein in nuclear extracts from many different types of cell lineages, it is likely that this factor plays a generalized role in transcription. Consistent with the findings

of Dorn et al. (1987b), it is likely that the factors which control the tissue-specific expression and IFN-γ-responsiveness of class II MHC antigen expression will be distinct entities from these generalized transcription factors.

In the interpretation of the data from the gene transfer studies, one must consider the potential influences that genetic manipulations may have on the outcome of the experiment. For example, the number of plasmids introduced into a cell and the site of plasmid insertion into host chromosomal DNA may directly influence the expression of sequences under study. Nonetheless, with these caveats in mind, the gene transfer studies suggest that discrete regulatory regions of DNA upstream of the coding sequences of class II MHC genes may be involved in their basal and inducible expression. The results of the transcription elongation analysis presented in Figure 19 support these observations and confirm the transcriptional nature of the induction of I-region gene expression by IFN-y in a normal cell type (i.e., not a cell that has been genetically manipulated). Furthermore, the transcriptional elongation studies support the use of a transcriptional approach to assess the effects of the inhibitors IFN-α/β and DEX. The data represented in Figure 20, generated from transcription elongation assays of macrophage cultures treated with IFN-γ and either IFN-α/β or DEX, demonstrate that both inhibitors block the transcriptional induction of I-region gene expression by IFN-γ. These data indicate that simultaneous treatment of macrophages with rIFN-γ and IFN-α/β results in an ~35% reduction in rate of transcription of I-region genes when measured 24 hr after treatment. Similarly, the simultaneous treatment of macrophages with rIFN-y and DEX results in an ~40% reduction in I-region gene transcription when measured 24 hr after treatment. Suppression becomes markedly more profound (> 50% for either IFN- α/β or DEX) when measured at 36 hr after treatment. These reductions in the levels of pre-initiated I-region transcripts are within the range necessary to account for the magnitude of the reduction in RNA accumulation previously reported in the steady-state inhibitor analysis (reviewed in Darnell, 1982). Given the apparent indirect nature of the induction of Ia antigen expression by

IFN-γ (Figure 7), it is impossible to determine from our studies if the inhibitors are acting directly at the I-region locus or indirectly at the trans-activating locus. There is evidence which suggests that IFN-α/β and DEX modulate changes in the transcriptional activity of genes. For example, IFN-\alpha treatment of neuroblastoma cells results in the transcription of the metallothionein-II gene and class I MHC antigen genes (Friedman and Stark, 1985). These IFN-α-inducible genes share a conserved sequence in which the heptanucleotide, AGTTTCT, is absolutely invariant. In addition, the class II MHC gene, HLA-DR contains this consensus sequence 567 bp upstream of the TATA box (Friedman and Stark, 1985). Although this sequence operates, presumably, as an activator of gene transcription, it is possible that similar sequences may act as "silencers" of transcription. This is consistent with the observation that the HLA-DR gene is poorly induced, if induced at all, by IFN-α (Rosa et al., 1983; Kelley et al., 1984). Similarly, IFN-β treatment of Ehrlich ascites tumor cells has been shown to increase the rate of transcription of a gene coding for a 56,000 dalton protein (Samanta et al., 1986). Within the first untranslated exon of this gene there is a sequence (partially homologous to the sequence identified by Friedman and Stark) which may play a role in the IFN-β inducibility of this gene.

DEX, bound together with the soluble glucocorticoid receptor, has been shown to bind upstream and within a number of glucocorticoid-regulated genes (Payvar et al., 1983; Renkawitz et al., 1984; Claverie and Sauvaget, 1985). These binding sites or glucocorticoid response elements (GRE's) typically have the core consensus sequence ACTGTTCTT and this sequence has been identified on the non-coding strand of the the Aβ gene at position -284 upstream from the cap site (Fertsch et al., 1987). Paradoxically, all of the published GRE's are for genes that are activated by glucocorticoids. However, Drouin and colleagues have identified a glucocorticoid-dependent "silencer" sequence (CGTCCA) upstream of the gene coding for pro-opiomelanocortin (POMC), a gene whose transcription is suppressed by glucocorticoids and activated by corticotropin releasing hormone (Charron and Drouin, 1986; Dr. J. Drouin, personal communication). This

sequence has also been found 883 nucleotides upstream from the cap site of the $E_{\beta}{}^d$ gene. The proof that these DNA sequences are involved in the modulation of class II MHC antigens directly or in the modulation of the putative trans-activating product will require detailed transfection studies of site-directed mutagenic constructs of sequences in this region. However, there is evidence from Arya et al. (1984) which demonstrates that DEX, at concentrations comparable to those used in this study, inhibits the accumulation of IFN- γ -specific mRNA in normal T lymphocytes. Therefore, in vivo DEX may modulate class II MHC antigen expression through its direct inhibitory effects on the transcription of these loci (or loci of trans-activating factors) and/or indirectly, through its inhibition of IFN- γ production.

In summary, the data derived from the steady-state and transcription rate analyses of Ia antigen expression arrived at in this study strongly support the hypothesis that both the induction of Ia antigen expression by rIFN-γ, as well as its antagonism by IFN-α/β and DEX, are transcriptionally regulated. Although the exact mechanisms of the induction and down-regulation of Ia antigen expression, with regard to the identity of second messengers and intermediate factors, are not fully elucidated, these studies have provided evidence for the role for a trans-activating intermediate(s) in this complex regulatory system. In studying the control of class II MHC antigen expression from both inductive and antagonistic vantages, we may be able to identify mechanisms which lead to the generation of self-reactivity. Does autoimmunity resulting from aberrant expression of class II MHC molecules on cells which do not normally express class II MHC molecules involve: (i) a hyperactivity of inducer-cell populations (those populations involved in the production of IFN-γ or other class II MHC antigen inducers); (ii) a hypoactivity of inhibitor-cell populations (those populations involved in the production of antagonists, such as glucocorticoids, IFN-α/β, PGEs); or, (iii) a combination of both which could lead to de-regulated control within the antigen-presenting cell? At the other extreme, can immunosuppression be explained at the level of a paucity of Ia antigen on cells that

normally present antigen? Immunosuppression in the neonate may be a normal mechanism to ensure that self-reactive populations remain unactivated. Lewis et al. (1986) demonstrated that there are reduced levels of IFN-y mRNA in human neonates. Wakasugi and Virelizier (1985) have shown that cord blood leukocytes, stimulated with mitogens, produce very low titers of IFN-y. However, this defect in IFN-y production could be reversed by treatment with indomethacin. Both observations suggest that the inductive signals involved in the regulation of class II MHC antigen expression might be impaired in neonates. Data presented above from Inaba et al. (1986), which showed that the failure of newborn macrophages to respond to IFN-y to express DR antigen could be reversed by the addition of anti-IFN-β antibody, suggest that the inhibitory signals involved in the regulation of class II MHC antigen expression might be exaggerated in neonates. Given the complex nature of the regulation of class II MHC antigen expression, from the early interactions of the inducer and receptor at the cell membrane to the late interactions which lead to the modulation of gene expression, investigators are attempting to identify relevant components. From these types of studies, it may be possible to identify the intermediates in complex regulatory cascades. With the elucidation of these intermediates and the mechanisms of gene modulation, we may be better equipped to intervene therapeutically and to prevent the perpetuation of ongoing autoimmune or immunosuppressive states.

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